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(57) Abstract

Liposomes which have present on their surface a polypeptide capable of binding to a mucosal cell surface of a human or animal and which are substantially free of active neuraminidase are useful as vaccines.

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VACCINES

This invention relates to liposomes, a process for their preparation, and pharmaceutical compositions containing them.

It is known that while a variety of inactivated viruses are good immunogens they are also pyrogenic which presents a serious disadvantage to their use as vaccines. One example of this are current influenza vaccines. These are composed of whole virus and suffer from problems of pyrogenicity as well as sensitization to egg proteins. An alternative is to use influenza vaccines composed of virus sub-units but these are poorly immunogenic and stimulate poor protection compared to live infection.

Generally, the poorest responses to influenza vaccines are observed in elderly patients who are most at risk from complications and death following infection with influenza. In addition to these problems, influenza vaccines are unpopular as they are conceived to be ineffective and because of fear of injections.

GB-A-1564500 discloses antigenic preparations containing a plurality of unilamellar microvesicles, otherwise known as virosomes, each microvesicle comprising a single lipid bilayer upon the exterior surface of which is bound an antigenic protein derived from a virus. GB-A-1564500 is related to two U.S. Continuation-in-Part Patents, US-A-4196191 and US-A-4148876. US-A-4148876 discloses antigenic virosome preparations of the type disclosed in GB-A-1564500 in which the antigenic protein is bound by hydrophobic bonding and is a haemagglutinin and neuraminidase sub-unit of a protective surface antigen derived from a myxovirus and having a hydrophobic region.

We have now found that influenza virosomes which comprise reconstituted virus envelopes and which have been treated to inactivate neuraminidase are highly immunogenic when administered intranasally. Significant IgA responses were observed in the lung lavage fluid of mice immunised

intranasally but not parenterally. These findings have general applicability. Accordingly, the present invention provides liposomes which have present on their surfaces a polypeptide capable of binding to a mucosal cell surface of a human or animal and which are substantially free of active neuraminidase. The liposomes are typically virosomes.

Liposomes are lipid vesicles enclosing a three-dimensional space. Envelope viruses comprise a lipid envelope. Liposomes according to the present invention may therefore be made of the lipid of an envelope virus. The virus envelope may be reconstituted after an envelope virus has been disrupted, for example by a detergent, thereby to form liposomes.

Useful liposomes may also be made of natural or synthetic phosphocholine-containing lipids having one fatty acid chain of from 12 to 20 carbon atoms and one fatty acid claim of at least 8 carbon atoms, for example 12 to 20 carbon atoms. Such lipids include dimyristoylphosphatidylcholine, dioleoylphosphatidylcholine, dipalmitoylphosphatidylcholine, dipalmitoylphosphatidylglycerol, distearoylphosphatidylcholine, phosphatidylcholine, phosphatidylserine and sphingomyelin. Another lipid may also be included in the liposomes, for example cholesterol, which is preferably present as less than 30% w/w of the whole lipid composition. The lipids may further comprise a material to provide a positive or negative charge, such as phosphatidic acid, dicetyl phosphate, phosphatidyl serine or phosphatidyl inositol to provide a negative charge or stearyl amine or other primary amines to provide a positive charge.

The liposomes used in the present invention may be either unilamellar or multilamellar, preferably unilamellar. They are typically biodegradable. The lipid of which they are composed is generally non-antigenic. The liposomes may encapsulate a substance, for example an antibody, antigen or drug. They may therefore be used as a delivery system for

the encapsulated component. The liposomes can be used as a general delivery system.

Typically the environment within the liposomes is an aqueous environment. A variety of substances can be encapsulated within the liposomes, such as peptides, proteins or adjuvants. The substance may be a substance against which it is wished to induce an immune response. Substances which may be encapsulated include antigenic subunits prepared from many types of virus such as herpes simplex virus, hepatitis A virus and hepatitis B virus. Proteins or peptides containing class 1 T-cell epitopes may be used. Encapsulation of this material within virosomes may help to generate a cytotoxic T-cell response against them.

The liposomes are preferably in a form which is suitable for intranasal administration. Preferably, therefore, the mucosal cell surface-binding polypeptide imparts on the liposomes the ability to bind to the nasal mucosa or to the mucosa of the lungs. Preferably the diameter of the liposomes is from 5 to 1000nm, for example 10 to 400 nm and most preferably from 20 to 100 nm.

The polypeptide capable of binding to a mucosal cell surface may be glycosylated or unglycosylated. The polypeptide may therefore be in the form of a glycoprotein. Preferably the polypeptide renders the liposomes fusogenic so that they are able to fuse with, rather simply bind to, host cell membranes. These membranes may be either the outer membrane of the membrane of endosomes following endocytosis. The polypeptide is typically a virus envelope polypeptide or is derived from a virus envelope polypeptide. All envelope viruses have a surface-binding function. The polypeptide may therefore be a polypeptide which is naturally present on the surface of an envelope virus and which provides the liposomes with the capability of binding to a cell surface. The virus may be a myxovirus such as influenza, mumps or measles virus. In particular the

polypeptide may be or be derived from an influenza virus envelope protein, for example of influenza virus type A, B or C.

The polypeptide capable of binding to a cell surface may for example be a haemagglutinin. Haemagglutinin is an integral membrane glycoprotein present in myxoviruses which is commonly composed of three monomers or sub-units. During infection of host cells, it serves two functions. Firstly, it attaches the virus to the cell by the binding of sialic acid residues present on cellular glycoproteins and glycolipids. Second, after internalization of virus into cellular endosomes the subsequent acidification triggers conformational changes in the haemagglutinin which lead to the fusion of viral and cellular membranes. Haemagglutinins are antigenic and stimulate the production of antibodies in hosts.

Another type of polypeptide capable of binding to a cell surface may be a bacterial adhesive protein such as the β-subunit of cholera toxin (CTB) or the heat-labile enterotoxin β-subunit of E. coli (LTB). This may also be used as an adjuvant in combination with haemagglutinin.

Neuraminidase is another glycoprotein which is found as an integral membrane protein in myxoviruses. This functions to cleave sialic acid residues and prevent the irreversible binding of virus to a host cell membrane by haemagglutinin. If active neuraminidase is present in the liposomes, then a significantly lower immunological response is observed. If active neuraminidase would otherwise be present in the liposomes, it must be inactivated. Neuraminidase may be inactivated by heat or by incubation with a neuraminidase inhibitor such as 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (DDAN).

The present liposomes are prepared by a process which comprises forming liposomes which have present on their surfaces a polypeptide capable of binding to a mucosal cell surface of a human or animal and which are

substantially free of active neuraminidase.

The polypeptide capable of binding to a cell surface may be added to the lipid materials before, during or after formation of the liposomes. Alternatively, virosomes can be prepared using the natural lipid of the envelope of an envelope virus to provide the necessary lipid component. If the polypeptide does not naturally associate with lipids it may be coupled to a fatty acid such as phosphatidylethanolamine (PE) by the use of a cross-linking agent such as succinimidyl 4-(p-maleimidophenyl)butyrate (SMPB).

Liposomes may for example be prepared by dissolving the lipid starting material in a solvent and evaporating the solvent. The lipid layer is then dispersed with aqueous saline or a buffer (if it is intended to incorporate the polypeptide into the liposomes after vesicle formation) or with an aqueous suspension of the polypeptide (if it is intended to form vesicles in the presence of the polypeptide). The dispersion is then agitated, for example by sonication. Polypeptide may then be added where it is not already incorporated in the surface of the liposomes and the vesicles again agitated.

An alternative method is to add the lipid starting material to an aqueous phase and slowly heat the mixture. It is then agitated to form liposomes. The aqueous phase may contain the polypeptide or it may be added subsequently.

A further method of preparing liposomes comprises the rapid injection of an ethanolic solution of lipid into aqueous saline or a buffer which has previously been purged with nitrogen. The resulting liposome preparation is then concentrated by ultrafiltration with rapid stirring under nitrogen at low pressure to avoid the formation of larger non-heterogeneous liposome. The ethanol may be removed from the vesicle fraction by analysis or washing with an ultra-filter. The polypeptide may be present in aqueous solution or alternatively the liposome fraction obtained after

ultrafiltration may be lightly sonicated with the polypeptide.

The liposome preparations obtained in the manner described above comprise aqueous dispersions of the lipid vesicles.

If the liposomes comprise neuraminidase then this must be inactivated. This may be achieved by heating the aqueous dispersion of liposomes comprising active neuraminidase to a temperature of for example from 30 to 60°C, for example from 50 to 60°C, more preferably from 53 to 58°C and most preferably about 55°C. The length of time required for neuraminidase inactivation will depend on the strain of virus and the temperature but is typically from 5 minutes to 5 hours, for example from 15 minutes to 3 hours. At low temperatures eg. 30°C a longer period of heating is required, whilst at higher temperatures a shorter period is required. We have found for influenza virus that heating at 55°C must be for 120 minutes or more, for example up to 180 minutes, in order to achieve an optimum effect. At 56°C, however, the optimal period for heating is from 6 to 10 minutes, for example about 8 minutes.

Alternatively, active neuraminidase may be deactivated by incubation of the liposomes with a neuraminidase inhibitor such as DDAN. As a further alternative active neuraminidase may be inactivated by heat or incubation with a neuraminidase inhibitor prior to incorporation into the liposomes.

A suitable way of preparing liposomes comprises:

(a) disrupting a myxovirus and removing the viral genome and internal viral protein or proteins; and

(b) forming liposomes in the presence of the material remaining, especially the envelope protein or proteins; and

(c) inactivating the neuraminidase present in the thus-formed liposomes.

Step (a) may be achieved be detergent solubilisation of viral particles and removal of internal

viral proteins and RNA. In an alternative way of preparing liposomes, the cell surface-binding polypeptide may be prepared by a recombinant DNA methodology. It will then necessarily be provided free of neuraminidase, so liposomes substantially free of active neuraminidase are necessarily obtained.

The liposomes of the present invention may be administered in the form of a pharmaceutical or veterinary composition which additionally comprises a suitable pharmaceutically or veterinarily acceptable carrier or diluent. The compositions are suitable for administration intranasally.

The compositions are preferably provided in a sterilised form. They may take the form of an aerosol. The compositions may further comprise preservatives, stabilisers and other conventional vaccine excipients if required.

The dosage of liposomes will vary depending upon a variety of factors. These include the nature of the cell surface-binding protein, the recipient (human or animal), the vaccination schedule and the extent of adjuvanticity conferred by the preparation. In general a dose of liposomes may be administered intranasally as a single unit or as a multiplicity of a sub-dosage over a period of time. Typically the unit dose for intranasal delivery to a human is from 2 to 500 μg.

We have also found that inactivated influenza virus which is substantially free of inactive neuraminidase is highly immunogenic when administered intranasally. This finding also has general applicability. The invention therefore further provides:

- an influenza virus which is not infectious and which is substantially free of active neuraminidase, for use as an influenza virus; and
- use of an influenza virus which is not infectious and which is substantially free of active neuraminidase in the preparation of a medicament for use as an influenza vaccine.

The influenza virus may be any influenza virus, for example type A, B or C. The virus is the virus against which it is wished to vaccinate. The neuraminidase may be inactivated by heating or specific inhibitors. An aqueous dispersion of the virus may be heated. Heating may be carried out at a temperature of for example from 30 to 60°C, more preferably from 53 to 58°C and most preferably about 55°C.

The length of time for which heating must be conducted to ensure neuraminidase inactivation will depend upon the strain of virus and the temperature but is typically from 5 minutes to 5 hours, for example from 15 minutes to 3 hours. At low temperatures, e.g. 30°C, a longer period of heating is required than at higher temperatures. We have found that heating at 55°C must be for 120 minutes of more, for example up to 180 minutes, in order to achieve an optimum effect. At 56°C, however, the optimal period for heating is from 6 to 10 minutes, for example 8 minutes.

The influenza virus is inactivated. In particular, viral infectivity is inactivated. This may be achieved by the heating to inactivate the neuraminidase. Typically, however, it is achieved by irradiation with ultraviolet light to provide a fail-safe inactivation procedure. This may be carried out before, simultaneously with or after treatment to inactivate the neuraminidase. Irradiation is carried out for at least 5 minutes, for example for from 5 to 60 minutes, at 400$\mu W/cm^2$ at a short wavelength, for example from 240 to 250 nm. The ultraviolet-inactivated, heated virus is grown in the allantoic fluid of embryonated hens eggs, for 2-3 days, recovered and purified on sucrose gradients.

The inactivated influenza virus substantially free of active neuraminidase is administered in the form of a pharmaceutical composition which additionally comprises a suitable pharmaceutically acceptable carrier or diluent.

The compositic . are suitable for administration intranasally.

The c mpositions are preferably provided in a sterilised form. They may take the form of an aerosol. The compositions may further comprise preservatives, stabilisers and other conventional vaccine excipients if required.

The dosage of inactivated virus will vary depending upon a variety of factors. An effective amount of the inactivated influenza virus substantially free from active neuraminadase is administered to a person in need of vaccination, in particular in need of vaccination against the said virus. Factors which need to be taken into account in assessing dosage include the age of the recipient, the vaccination schedule and the extent of adjuvanticity conferred by the preparation. In general a dose may be administered intranasally as a single unit or as a multiplicity of a sub-dosage over a period of time. Typically the unit dose for intranasal delivery is from 2 to 500 μg.

The vaccines of the invention exhibit advantages over current influenza vaccines. These include immunogenicity, the convenience of intranasal administration and the production of local mucosal immunity.

The invention will now be further illustrated by means of the following Example. In the accompanying drawings:

Figure 1A shows the ELISA titres against X31 influenza virus in sera from Balb/c mice immunised intranasally (i.n.) with heated and acid-treated virosomes or virus;

Figure 1B shows the ELISA titres against denatured virus in sera from mice immunised i.n. with heated and acid-treated virosomes or virus;

Figure 1C shows the neutralisation titres of sera from mice immunised i.n. with heated and acid-treated virosomes or virus;

Figure 1D shows the HAI titres against virus in sera from mice immunised i.n. with heated and acid-treated virosomes or virus;

Figure 2A shows the ELISA titres against virus in sera from mice immunised i.n. with heated and acid-treated virus;

Figure 2B shows the neutralisation titres against virus in sera from mice immunised i.n. with heated and acid-treated virus;

Figure 3A shows the ELISA titres against virus in sera from mice immunised i.n. with heated and acid-treated virosomes;

Figure 3B shows the neutralisation titres against virus in sera from mice immunised i.n. with heated and acid-treated virosomes;

Figure 4A shows the ELISA titres against virus in sera from mice immunised i.n. with heated and acid-treated virosomes encapsulating the internal proteins and RNA ("virosome-cores");

Figure 4B shows the neutralisation titres against virus in sera from mice immunised i.n. with heated and acid-treated virosome-cores;

Figure 5A shows the ELISA titres against virus in sera from mice immunised i.n. with heated and acid-treated virosomes encapsulating ovalbumin ("ova-virosomes");

Figure 5B shows the neutralisation titres against virus in sera from mice immunised i.n. with heated and acid-treated ova-virosomes;

Figure 6A shows the individual ELISA titres against virus in sera (2 days post-challenge bleed) from mice immunised i.n. with heated and acid-treated virus;

Figure 6B shows the individual neutralisation titres against virus in sera (2 days post-challenge bleed) from mice immunised i.n. with heated and acid-treated virus;

Figure 7A shows the individual ELISA titres against

virus in sera (2 days post-challenge bleed) from mice immunised i.n. with heated and acid-treated virosomes;

Figure 7B shows the individual neutralisation titres against virus in sera (2 days post-challenge bleed) from mice immunised i.n. with heated and acid-treated virosomes;

Figure 8A shows the individual ELISA titres against virus in sera (2 days post-challenge bleed) from mice immunised i.n. with heated and acid-treated virosome cores;

Figure 8B shows the individual neutralisation titres against virus in sera (2 days post-challenge bleed) from mice immunised i.n. with heated/acid-treated virosome-cores;

Figure 9A shows the individual ELISA titres against virus in sera (2 days post-challenge bleed) from mice immunised i.n. with heated and acid-treated ova-virosomes;

Figure 9B shows the individual neutralisation titres against virus in sera (2 days post-challenge bleed) from mice immunised i.n. with heated/acid-treated ova-virosomes;

Figure 10A shows ELISA titres showing the effect of heating on the immunogenicity of virosomes administered i.n.;

Figure 10B shows the neutralisation titres showing the effect of heating on the immunogenicity of virosomes administered i.n.;

Figure 11 compares the anti-virus and neutralising antibody response following immunisation with heated virosomes.

Figure 12A shows the effect of heating at 55°C on the immunogenicity of virosomes administered i.n.;

Figure 12B shows the effect of heating at 55°C on the immunogenicity of virosomes administered i.n.;

Figure 13 shows the effect of pre-treating virosomes or mice with gangliosides on the immunogenicity of virosomes given i.n.;

Figure 14A shows the ELISA results of sera from mice immunised i.n. on days 0 and 43 with different doses of influenza virosomes; and

Figure 14B shows the neutralisation results of sera from mice immunised i.n. on days 0 and 43 with different doses of influenza virosomes.

EXAMPLE

1. METHODS

Preparation of virosomes

The procedure for making the reconstituted virus envelopes was similar to that described by Metsikko et al. (EMBO J. 5, 3429-3435, 1986) and Stegmann et al. (EMBO J. 6, 2651-2659, 1987). A pellet of X31 influenza virus (5mg) was solubilised in 0.7ml of 100mM octaethyleneglycol monododecylether ($C_{12}E_8$) in dialysis buffer (145mM NaCl, 5mM Hepes, pH 7.4) for 20 min at room temperature. The mixture was centrifuged at 170,000g from 30 min to remove the internal proteins and RNA. 0.56ml of the supernatant was added to 160mg of wet Bio-Beads SM-2 and shaken on a rotating table (approx. 400 rpm) for 1 hour at room temperature. The supernatant was removed from the beads with a 23g needle attached to a 1ml syringe and added to 80mg of wet Bio-Beads SM-2 and shaken on a rotating table (approximately 500-600rpm) for 8 min yielding a turbid suspension. The supernatant was removed with a 23g needle and syringe. The virosomes were separated from unincorporated protein by discontinuous sucrose gradients (40%/5% or 40%/20%/5%) spun at 170,000g for 90min. The morphology of the virosomes was analysed by electron microscopy using negative staining with phosphotungstate.

Virosomes containing encapsulated proteins, e.g. ovalbumin (Virosomes + ova), were made as described above except that 100µl of 200 mg/ml ovalbumin was added prior to adding the SM-2 beads. Virosomes-cores were made as described above except that the interal proteins and RNA

were not removed by centrifugation.

ELISA assays

Anti-virus antibodies in the serum from vaccinated mice were measured by ELISA (enzyme-linked immunoadsorbent assay). The virus antigen was diluted in carbonate coating buffer pH 9.5: 1/50 dilution of allantoic fluid from hens eggs inoculated with virus or 1µg/ml of purified egg-grown virus. Microtitre plates were coated with antigen and left at 37°C for 1 hour and then overnight at 4°C. After washing the plates 3 times in 0.05% Tween 20 in PBS 100µl of 1% BSA was added and left at 37°C for 1 hour to block the plates. The antisera to be tested was diluted down or across the plate in doubling or half log dilutions in 1% BSA in PBS and left at 4°C overnight. The plates were washed with Tween/PBS before adding the enzyme-conjugated second antibody at 1/500-1/1000 in 1% BSA in PBS. The plates were left at 37°C for 2 hours and washed in Tween/PBS. The substrate, o-phenylenediamine dihydrochloride (OPD) (10mg/100ml) in citrate buffer with 0.01% H_2O_2 was added to the plates and the reaction stopped in H_2SO_4. The plates were read on a microplate reader at 492nm. The titres were end point titres determined by taking the titre at which the OD value was equal to the mean OD value obtained with 1/10 dilution of control normal sera plus 2 standard deviations.

In Vitro Neutralisation Assay

We have established a microtitre plate-based neutralisation assay on MDCK cells. Serial dilutions of antibody were incubated with 2 logs of virus for 1 hour at 37°C. These were transferred to microtitre plates with 70-90% confluent MDCK cells in MEM media without serum. After incubation at 37°C for 1 hour the supernatant was removed and fresh MEM added with 10µg/ml trypsin. The plates were stained after 48-72 hours and the neutralisation titres read by eye.

H&I - Haemagglutination inhibition assay

Haemagglutination and haemagglutination inhibition assays were performed as described by Fazekas de St. Groth and Webster, (J. Exp. Med. 124; 331-345, 1966).

Experiments

Experiments were carried out as follows, referring to the Figures:

Figure 1

Dose - 5μg of X31 virus/virosomes per mouse in 30μl volume given i.n.
All virosomes were uv. inactivated for 5 min (400μW/cm^2)
Heating - heating carried out at 55°C for 20 min.
Acid treatment - 1/100th volume of 3M acetate buffer pH 4.8 was added to the virosomes. These were left at 37°C for 15 min before neutralising the acid with 1M Tris pH 7.5.
Second immunization - 6 weeks
Bleed tested - 12 weeks

Figures 2-5

Dose - 5μg of X31 virus/virosomes per mouse in 30μl volume given i.n.
Virosomes were uv inactivated for 5 min (400μW/cm^2)
Heating - heating carried out at 55°C for 20 min.
Acid treatment - 1/100th volume of 3M acetate buffer pH 4.8 was added to the virosomes. These were left at 37°C for 15 min before neutralising the acid with 1M Tris pH 7.5.
Second immunization - 6 weeks

Figures 6-9

Dose - 5 μg of X31 virus/virosomes per mouse in 30μl volume given i.n.
Virosomes were uv inactivated for 5 min (400μW/cm^2)
Heating - heating carried out at 55°C for 20 min.

Acid treatment - 1/100th volume of 3M acetate buffer pH 4.8 was added to the virosomes. These were left at 37°C for 15 min before neutralising the acid with 1M Tris pH 7.5.
Second immunization - 6 weeks
Bleed tested - 12 weeks

Figure 10

Dose - 3 μg of X31 virosomes per mouse in 30μl volume given i.n.
Virosomes were uv inactivated for 5 min (400μW/cm^2)
Heating - heating carried out at 55°C for specified times.
Second immunization - 6 weeks

Figure 11

Dose - 3 μg of X31 virosomes per mouse in 30μl volume given i.n.
Virosomes were uv inactivated for 5 min (400μW/cm^2)
Heating - heating carried out at 55°C for specified times.
Second immunization - 6 weeks
Bleed tested - 8 weeks

Figure 12

Dose - 3μg of X31 virosomes per mouse in 30μl volume given i.n.
Virosomes were uv inactivated for 5 min (400μW/cm^2)
Heating - heating carried out at 55°C for specified times.
Second immunization - 6 weeks
Bleed tested - 12 weeks

Figure 13

preincubation with gangliosides and antibody

The virosomes were dialysed against Hepes buffer (145mM NaCl, 5mM Hepes pH 7.4 plus 3mM EDTA). These virosomes were heated at 55°C for 1 hour.
Dose - 3μg/mouse in 30μl volume.

Incubation with gangliosides - Virosomes were incubated at

37°C for 1 hour and then overnight at 4°C with a 12 Molar excess of gangliosides to viral haemagglutinin.

Pretreatment of mice with gangliosides - 100 Molar excess of gangliosides to viral haemagglutinin.

Incubation with antibody - 20μg of virosomes were incubated in 40μg of purified HC2 antibody or 20μg HC2 Fab fragments for 2 hours at 37°C

Administration of virosomes with CTB

2μg of CTB (B-subunit of cholera toxin) was given together with 3μg of virosomes to each mouse.

Second immunization - 6 weeks
Bleed tested - 12 weeks

Treatment with DDAN

20μg of virosomes were incubated with 1mM DDAN for 1 hour at 37°C and then at 4°C overnight.
dose per mouse = 3μg in 30μl volume i.n.
Second immunization - 6 weeks
Bleed tested - 12 weeks

Figure 14 (Dose response)

Dose - variable dose of X31 virosomes in 30μl volume given i.n.
Virosomes were uv inactivated for 5 min ($400\mu W/cm^2$)
Heating - heating carried out at 55°C for specified times.
Second immunization - 6 weeks

2. RESULTS

Effect of acid-treatment on the immunogenicity of virus and virosomes

Influenza virus, influenza virosomes, or influenza virosomes containing cores (HBcAg) or ovalbumin were treated

with acid (pH 4.8) for 30 min. at 37°C. Acid-treatment of virus or virosomes led to a dramatic reduction in immunogenicity of virus or virosomes given by the intranasal route as assayed by serum ELISA titres against native virus (Figures 1A and 2A-5A). This was not due to the fact that acid destroys some of the neutralisation epitopes on haemagglutinin because lower responses were also observed when the sera were tested against SDS-denatured virus (Figure 1B). In addition, the levels of neutralising antibodies induced were considerably reduced if the inoculum was acid-treated (Figure 1C and 2B-5B).

There appeared to be some protection against acid-inactivation of virosomes containing cores but this may be due to insufficient acidification of the boost inoculum (see Figure 4A & B). When the response of individual animals was analysed there was a consistent reduction in response if the virus or virosomes were acid treated (Figures 6 - 9). We also looked at the haemagglutination inhibition (HI) activity of the sera (Figure 1D), which also show a reduction in the titre of antibody stimulated when virosomes were acid treated before inoculation.

Acid-treatment (pH 4.8) of virus abrogates the ability of virus to fuse with cells while virus attachment is unaffected. This is due to the irreversible conformational shift in the conformation of haemagglutinin that normally occurs inside the endosome after uptake of the virus within coated pits. These results suggest that the virus or virosomes must not only bind to the mucosal surfaces but also fuse with the epithelial cells to stimulate optimal responses.

Effect of heating at 55°C on the immunogenicity of virus

Mice inoculated intranasally with X31 influenza virus heated for 20 min. showed significantly greater serum ELISA, HAI or neutralising antibody titres than mice receiving unheated virus (Figures 1A-D). Both the ELISA

titres against native virus and the neutralising titres were approaching those observed following immunization with the same dose of infectious virus (Figure 2). In a further experiment virus was heated for only 8 mins. and again this led to an increase in response following intranasal inoculation (Table 1).

Responses to both the heated or infectious virus were observed at least 21 days before responses to inactivated virus. When the response of individual animals was examined there was an increase in response when the virus was heated and the ELISA titres paralleled the neutralising titres (Figure 6). There was, however, considerable variation probably due to the efficiency of inoculation.

Effect of heating at 55°C on the immunogenicity of virosomes

In a preliminary experiment, mice were inoculated intranasally with virosomes, or virosomes containing cores or ovalbumin, that had been heated for 20 min. These heated virosomes stimulated comparable or greater serum ELISA or neutralising titres than mice receiving unheated virosomes (Figures 1-5). Figure 4 shows that uv-inactivated, heated virosomes containing viral cores stimulate a much earlier response than uv-inactivated or acid-treated virosomes. In addition, when the response of individual animals was examined there was little variation within the animal groups (Figure 7). The neutralisation titres showed greater variation but parallelled the ELISA titres. It should be noted that these virosomes were stored at 4°C, so it is possible that much of the neuraminidase activity was lost.

Mice were immunized with fresh virosomes that were stored in 50% glycerol at -20°C. A dramatic increase in immunogenicity was observed if the virosomes were heated for up to 128 mins. (Figures 10A and 11, Table 1). The levels of neutralizing antibodies showed a more dramatic increase with increasing periods of heating (Figures 10B and 11).

Animals immunized with unheated virosomes had undetectable levels of neutralising antibodies suggesting that the high responses observed in the previous experiments with unheated virosomes were due to partial inactivation of neuraminidase activity during storage at 4^0C. In addition, when serum from individual mice were analyzed a significant increase in ELISA and neutralising antibody titre was observed in sera from mice receiving virosomes heated for increasing periods of time (Table 2, Figure 12).

Effect of Specific Inactivation of Neuraminidase on the Immunogenicity of Virosomes Given Intranasally

We have carried out an experiment to determine whether the increase in immunogenicity observed with heating of virosomes was due to inhibition of neuraminidase (NA). Thus, the NA in virosomes was specifically inactivated with the neuraminic acid analogue, DDAN. DDAN-treated virosomes stimulated a greater response than untreated virosomes (log titre of 2.2 cf 1.6) showing that inhibition of neuraminidase leads to an increase in immunogenicity of intranasally administered virosomes.

Effect of Specific Blocking of Virosome Attachment by Pre-incubation with Gangliosides on the Immunogenicity of Virosomes given Intranasally

In order to study the effect of blocking virosome attachment on the immunogenicity of intranasally administered virosomes we have pre-incubated virosomes in various sialic acid-containing gangliosides. The immunogenicity of influenza virosomes administered intranasally (i.n.) could be partially abrogated by pre-treating the virosomes with GM1 or GD1a gangliosides but not by pre-treating with GT1b or a ganglioside mixture (Figure 13). Similarly, we have found that the virosome-mediated haemagglutination was partially inhibited only by the GM1 and GB1a gangliosides. These experiments show that binding

of virosomes to sialic acid receptors is critical for their immunogenicity.

Effect of pre-treating mice with gangliosides on the response of mice to virosomes given intranasally

We have studied the effect on the response to virosomes of increasing the viral receptors on the respiratory mucosal surfaces through intranasal pre-treatment of mice with various gangliosides (Figure 13). Pre-treatment with GM1 ganglioside but not GD1a GT1b or a ganglioside mixture led to an increase in response presumably because of an increase in density of receptors or replacement with higher affinity receptors on the mucosal surfaces facilitating greater binding and uptake of the virosomes.

Effect of Specific Blocking of Virosome Attachment by Pre-incubation with Neutralising Monoclonal Antibodies on the Immunogenicity of Virosomes Given Intranasally

Pre-incubation of virosomes with a neutralising monoclonal antibody (either whole or Fab fragments) completely inhibited haemagglutination. However, the immunogenicity of the virosomes was unaffected by prior incubation of virosomes in whole antibody or Fab fragments or i.n. inoculation of Fab fragments 2 hours after inoculation with untreated virosomes. This result is surprising and may indicate that some neutralising antibodies do not prevent virus binding or entry into mucosal epithelia but some later event (e.g. secondary uncoating). Studying the fate of antibody-treated virus or virosomes should provide some insight into the mechanisms of humoral immunity in the respiratory tract. Moreover, with regards intranasal vaccination of humans, it is encouraging if the presence of local neutralising antibody fails to reduce the immunogenicity of intranasally-administered virosomes.

Effect of Administering the B-subunit of Cholera Toxin (CT-B) Intranasally Together with Virosomes on the Immunogenicity of the Virosomes

We investigated whether CTB could enhance the responses to virosomes administered intranasally. Figure 13 shows that there was a dramatic increase (10-fold) in response to the virosomes when given with CTB).

Dose response study of virosomes administered intranasally

We have studied the response to a range of doses of virosomes administered intranasally. These virosomes were unheated and fresh so the antibody responses are relatively low. A clear dose response effect was observed with the minimal immunogenic dose being 1μg (Figure 14). We have repeated this experiment using heated virosomes which we would expect to be much more immunogenic.

Protection against Challenge

All the animals receiving intranasal immunisations of virus or virosomes have been challenged with live virus and the lungs were removed 2 days later. Preliminary challenge lung titres indicate that animals immunised with virus or virosomes that were not acid-treated were completely protected against infection.

Table 1 - INTRANASAL IMMUNISATION OF BALB/C MICE WITH INFLUENZA VIROSOMES

Effect of heating at 55°C on immunogenicity of virosomes

Group	Antigen	Heat	Dose	ELISA TITRE (OD)
63A	VIROSOMES	Not heated	3µg	1.90 (0.25)
63B	VIROSOMES	heated 2min	3µg	2.02 (0.25)
63C	VIROSOMES	heated 8min	3µg	2.52 (0.43)
63D	VIROSOMES	heated 32min	3µg	2.80 (0.54)
63E	VIROSOMES	heated 128min	3µg	3.09 (0.64)
63F	VIRUS	Not heated	3µg	2.44 (0.44)
63G	VIRUS	heated 8min	3µg	2.87 (0.77)
63H	in. PBS	control		<1.50 (0.17)

Bleed tested: 23/2/90 - 4 weeks after primary immunisation

Table 2 - INTRANASAL IMMUNISATION OF BALB/C MICE WITH INFLUENZA VIROSOMES - RESPONSE OF INDIVIDUAL MICE

Effect of heating at 55°C on Immunogenicity

Group	Antigen	Heat	Animal	ELISA TITRES NV	ELISA TITRES DV	MEAN TITRE NV	MEAN TITRE DV
63A	VIROSOMES	Not heated	G	1.21	1.23		
			Y	1.98	1.89		
			M	1.26	1.14	1.84	1.44
			W	1.48	1.23		
			R	1.31	1.10		
63B	VIROSOMES	heated 2min	M	<1.00	<1.00		
			W	1.38	1.02		
			G	2.02	1.38	1.64	1.24
			R	1.24	1.22		
			Y	1.78	1.42		

Group	Antigen	Heat	Animal	ELISA TITRES NV	ELISA TITRES DV	MEAN TITRE NV	MEAN TITRE DV
63C	VIROSOMES	heated 8min	R	2.26	1.99		
			Y	1.21	1.06		
			G	1.72	1.81	1.95	1.69
			W	1.86	1.80		
			M	2.12	1.13		
63D	VIROSOMES	heated 32min	M	2.33	1.75		
			Y	2.22	2.28		
			R	1.78	2.16	2.12	2.04
			G	2.25	2.19		
			W	1.65	1.07		
63E	VIROSOMES	heated 128min	R	2.33	2.33		
			Y	2.59	2.24		
			M	1.84	1.51	2.31	2.46
			W	1.88	1.48		
			G	2.47	2.99		

Bleed tested: 30/3/90 - 4 weeks after second immunisation.

CLAIMS

1. Liposomes which have present on their surface a polypeptide capable of binding to a mucosal cell surface of a human or animal and which are substantially free of active neuraminidase.

2. Liposomes according to claim 1, in which the haemagglutinin is a haemagglutinin a myxovirus.

3. Liposomes according to claim 2, in which the myxovirus is influenza, mumps or measles virus.

4. Liposomes according to claim 1, in which the polypeptide is a bacterial adhesion polypeptide.

5. Liposomes according to any one of the preceding claims which encapsulate a physiologically active substance.

6. Liposomes according to claim 5, wherein the substance is a peptide, protein or adjuvant.

7. A process for the preparation of liposomes according to any one of the preceding claims, which process comprises forming liposomes which have present on their surfaces the said polypeptide and which are substantially free of active neuraminidase.

8. A process according to claim 7, which comprises:
(a) disrupting a myxovirus and removing the viral genome and internal vital protein or proteins;
(b) forming liposomes in the present of the material remaining;
(c) inactivating the neuraminidase present in the thus-formed liposomes.

9. A process according to claim 8, wherein the neuraminidase is inactivated by heat or by incubation with neuraminidase inhibitor.

10. A process according to claim 9, in which the inactivation is achieved by heating to a temperature from 50 to 60 °C, or by incubation with 2,3-dehydro-2-deoxy-N-acetylneuraminic acid.

11. A process according to claim 7, wherein liposomes are formed using a said polypeptide which is

recombinant polypeptide.

12. A pharmaceutical composition which comprises liposomes according to any one of claims 1 to 6 in association with a pharmaceutically acceptable carrier or diluent.

13. A composition according to claim 12 which is in a form suitable for intranasal administration.

14. An influenza virus which is not infectious and which is substantially free of active neuraminidase, for use in a method of treatment of the human or animal body by therapy.

15. A virus according to claim 14, for use an influenza vaccine.

16. A virus according to claims 14 or 15, which has been heated to inactivate the neuraminidase.

17. A virus according to claim 16, wherein the said heating has been conducted at a temperature of from 50 to 60°C.

18. A virus according to any one of claims 14 to 17, which has been rendered non-infectious by treatment with ultraviolet light.

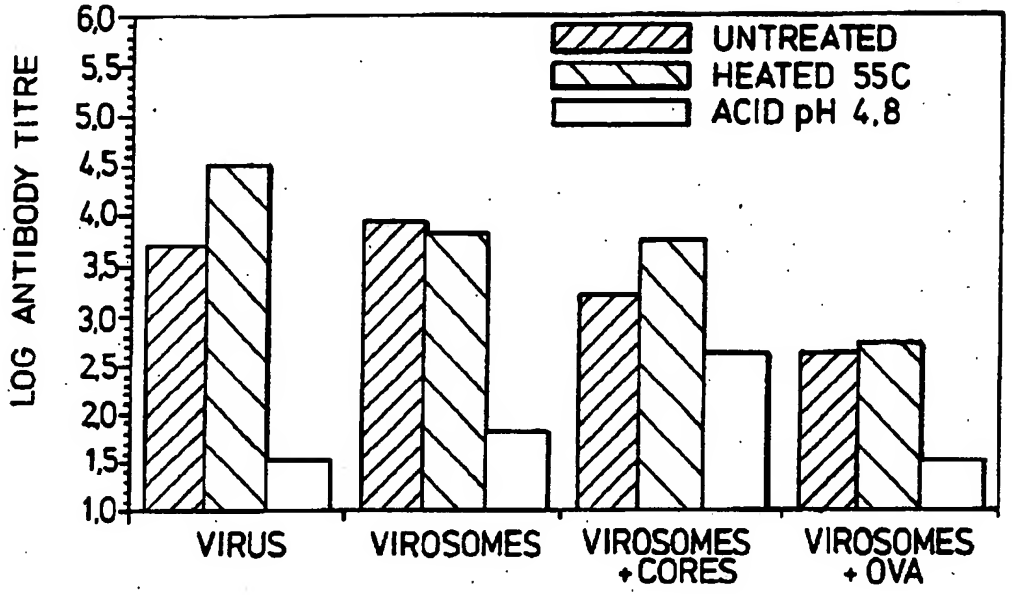
19. Use of an influenza virus which is not infectious and which is substantially free of active neuraminidase in the preparation of a medicament for use as an influenza vaccine.

20. Use according to claim 19, wherein the virus has been heated to inactivate the neuraminidase.

21. Use according to claim 20, wherein the said heating has been conducted at a temperature of from 50 to 60°C.

22. Use according to any one of claims 19 to 21, wherein the virus has been rendered non-infectious by treatment with ultraviolet light.

Fig.1A.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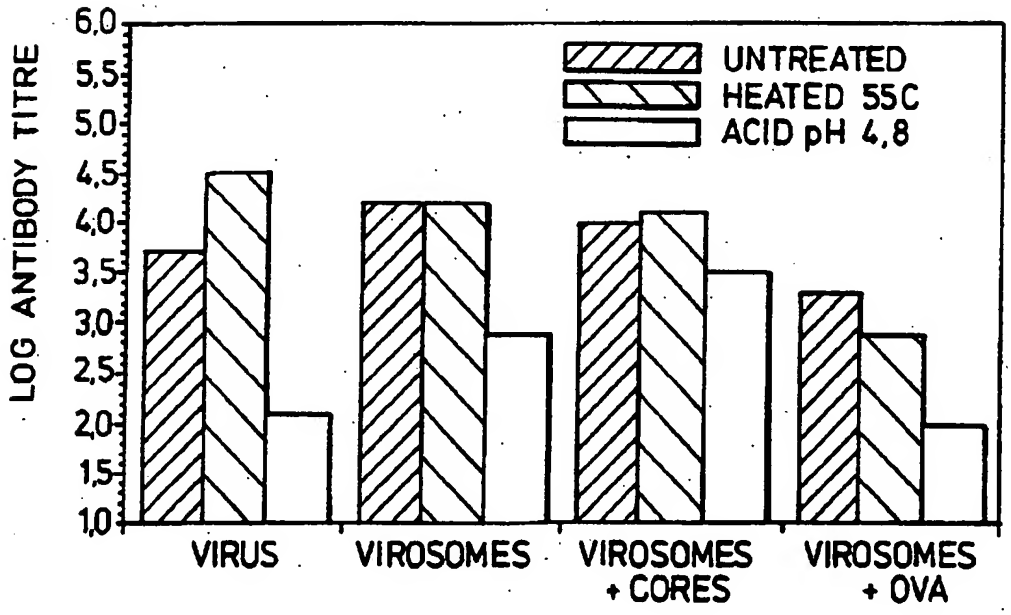
1,5
1,0
UNTREATED
HEATED 55C
ACID pH 4,8
VIRUS
VIROSOMES
VIROSOMES + CORES
VIROSOMES + OVA

Fig.1B.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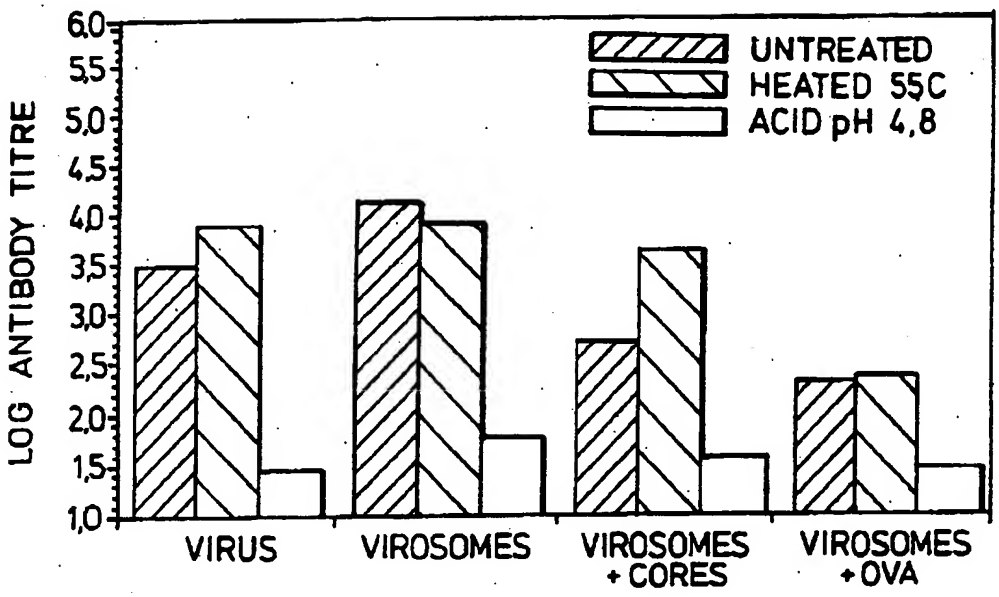
1,0
UNTREATED
HEATED 55C
ACID pH 4,8
VIRUS
VIROSOMES
VIROSOMES + CORES
VIROSOMES + OVA

Fig.1C.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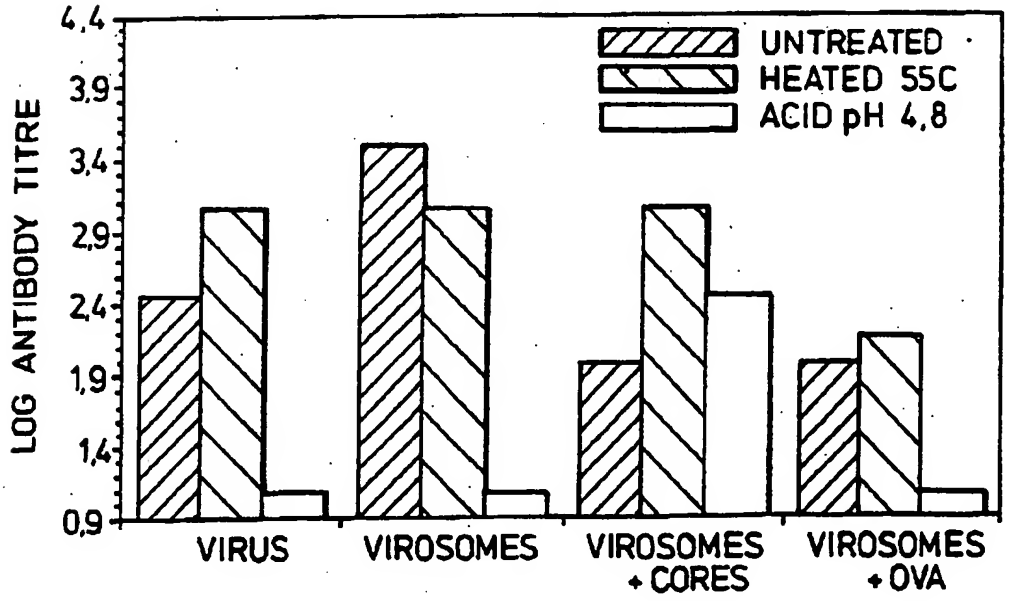
1,0
UNTREATED
HEATED 55C
ACID pH 4,8
VIRUS
VIROSOMES
VIROSOMES + CORES
VIROSOMES + OVA

Fig.1D.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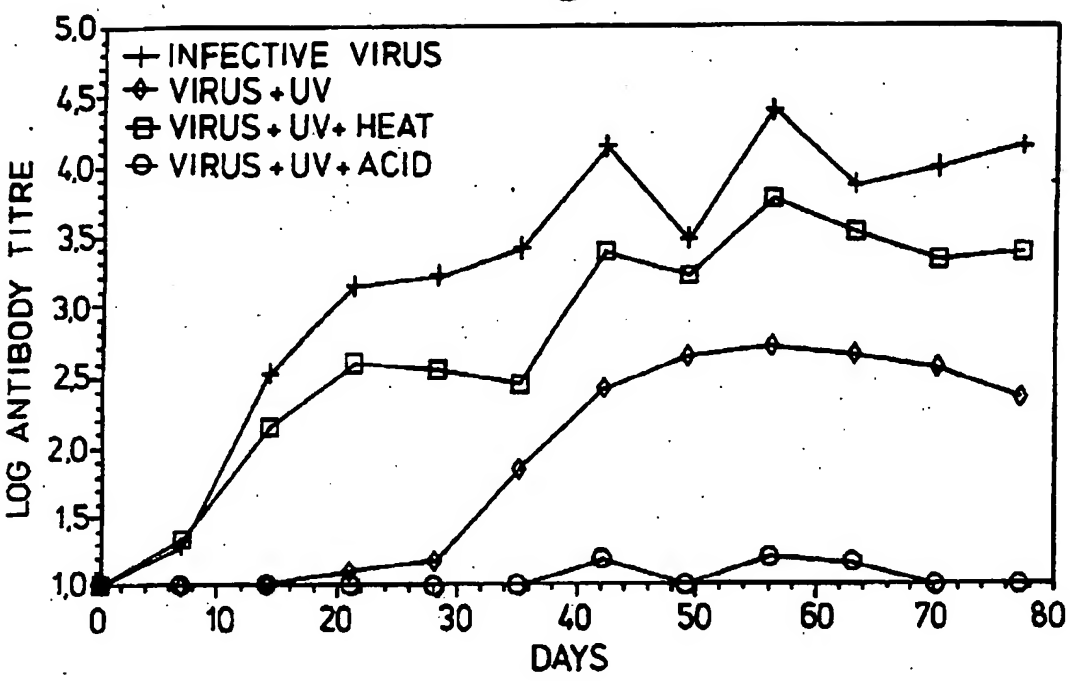
2,9
2,4
1,9
1,4
0,9
UNTREATED
HEATED 55C
ACID pH 4,8
VIRUS
VIROSOMES
VIROSOMES + CORES
VIROSOMES + OVA

Fig.2A.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VIRUS + UV
VIRUS + UV + HEAT
VIRUS + UV + ACID
LOG ANTIBODY TITRE
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4.0
3.5
3.0
2.5
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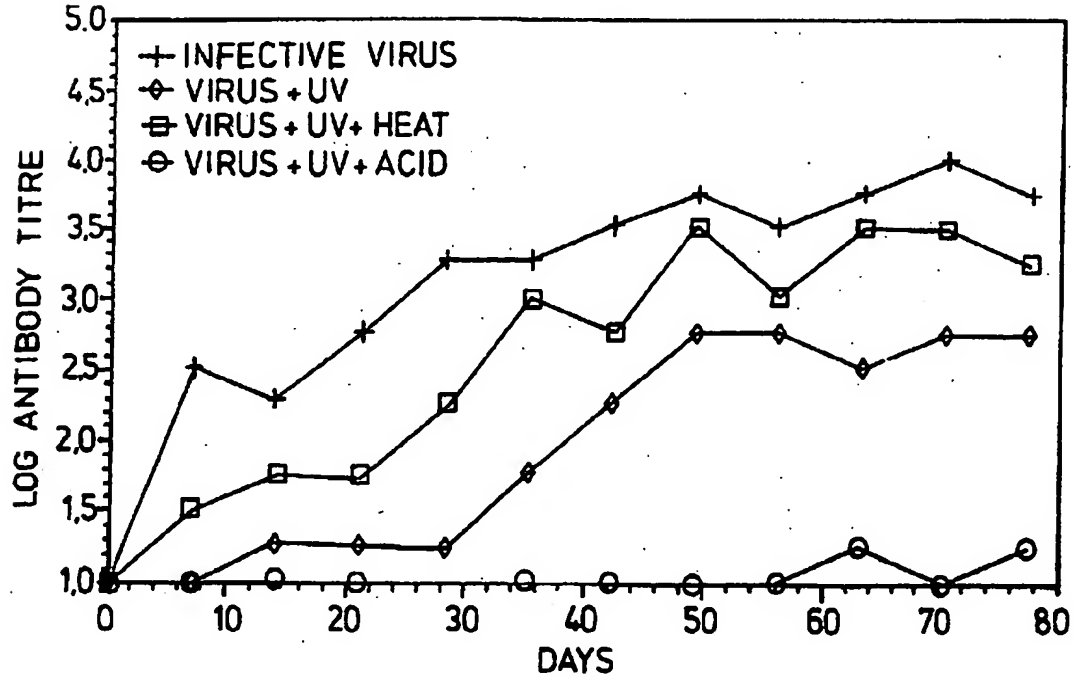
1.5
1.0
0
10
20
30
40
50
60
70
80
DAYS

Fig.2B.


INFECTIVE VIRUS
VIRUS + UV
VIRUS + UV + HEAT
VIRUS + UV + ACID
LOG ANTIBODY TITRE
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4.0
3.5
3.0
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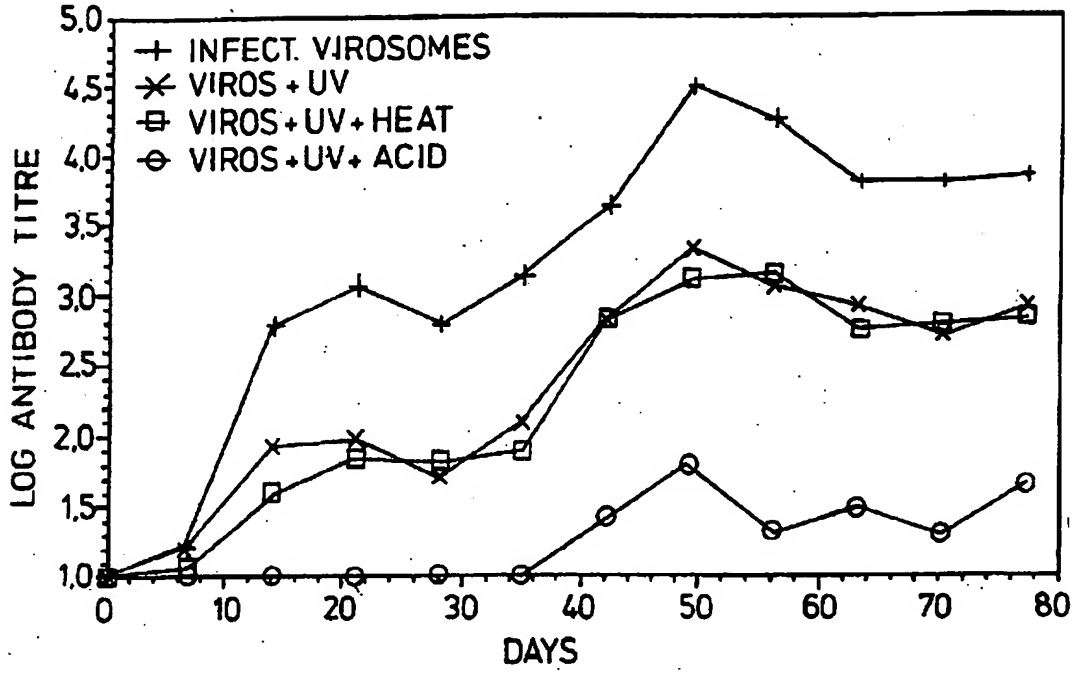
1.5
1.0
0
10
20
30
40
50
60
70
80
DAYS

Fig. 3A


INFECT. VIROSOMES
VIROS + UV
VIROS + UV + HEAT
VIROS + UV + ACID
LOG ANTIBODY TITRE
DAYS
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4.0
3.5
3.0
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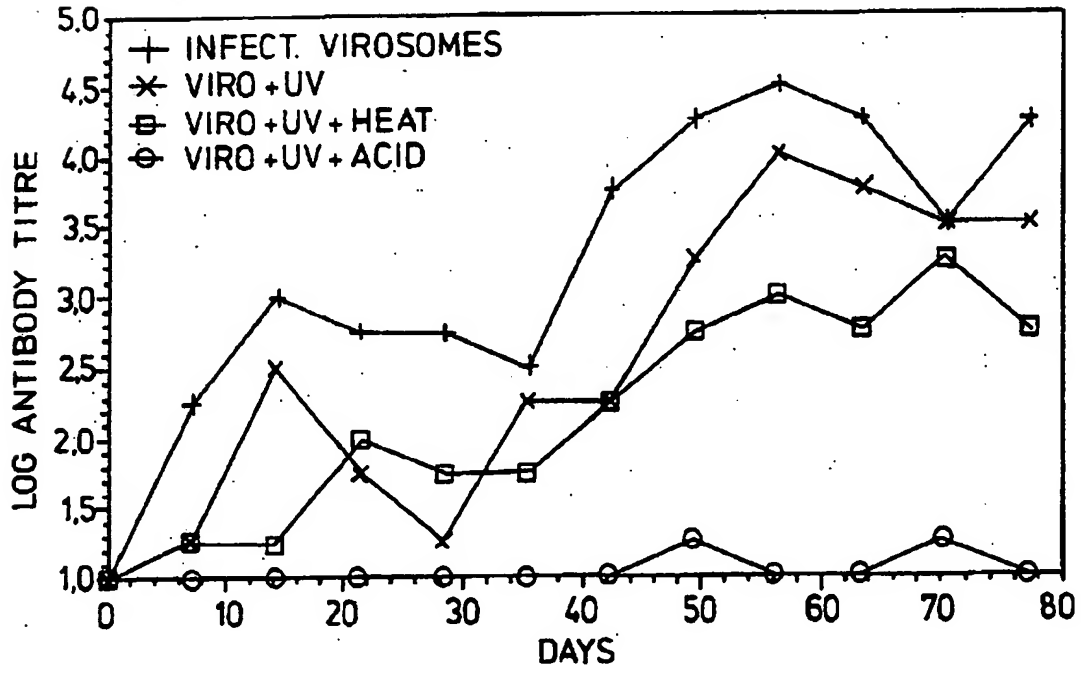
20
30
40
50
60
70
80

Fig. 3B.


INFECT. VIROSOMES
VIRO + UV
VIRO + UV + HEAT
VIRO + UV + ACID
LOG ANTIBODY TITRE
DAYS
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4.0
3.5
3.0
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1.5
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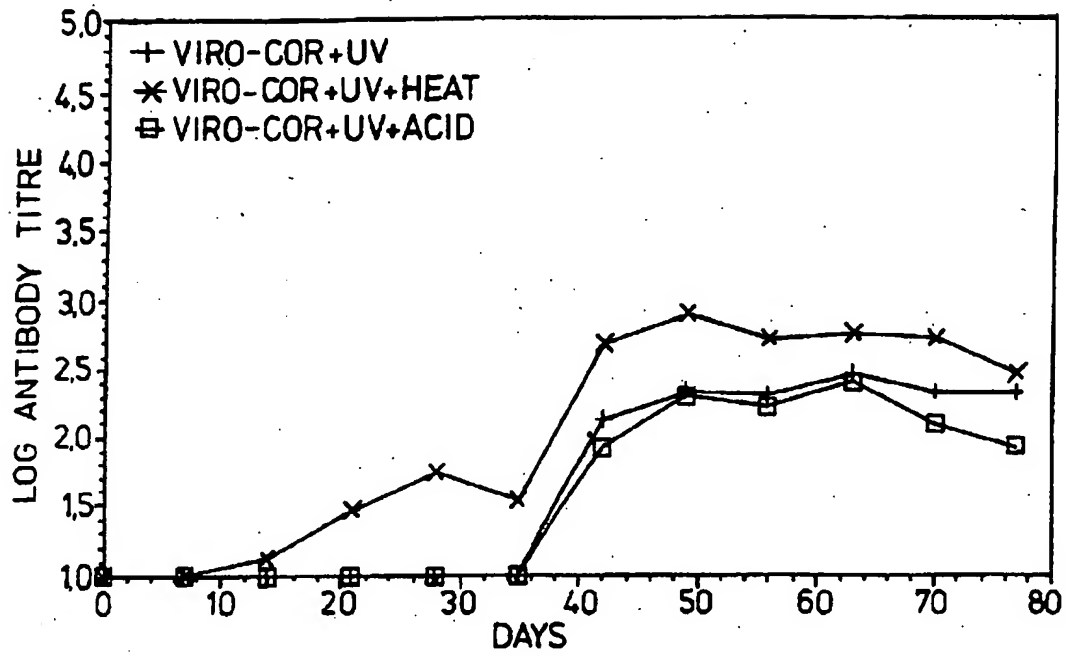
0
10
20
30
40
50
60
70
80

Fig.4A.


VIRO-COR+UV
VIRO-COR+UV+HEAT
VIRO-COR+UV+ACID
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3,5
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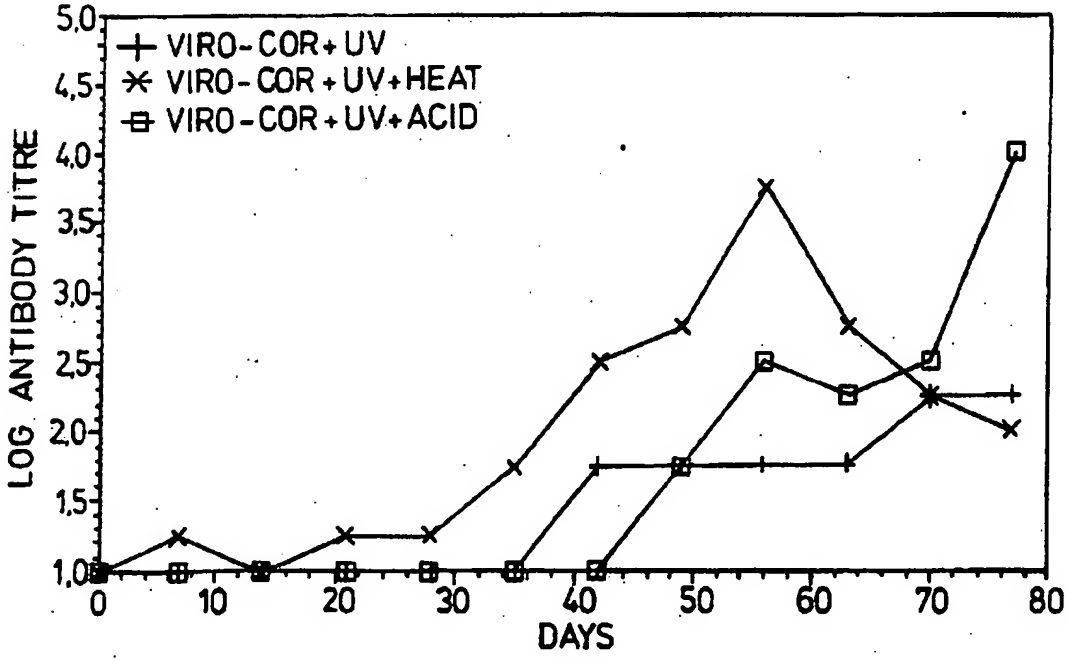
0
10
20
30
40
50
60
70
80
DAYS

Fig.4B.


VIRO-COR+UV
VIRO-COR+UV+HEAT
VIRO-COR+UV+ACID
LOG ANTIBODY TITRE
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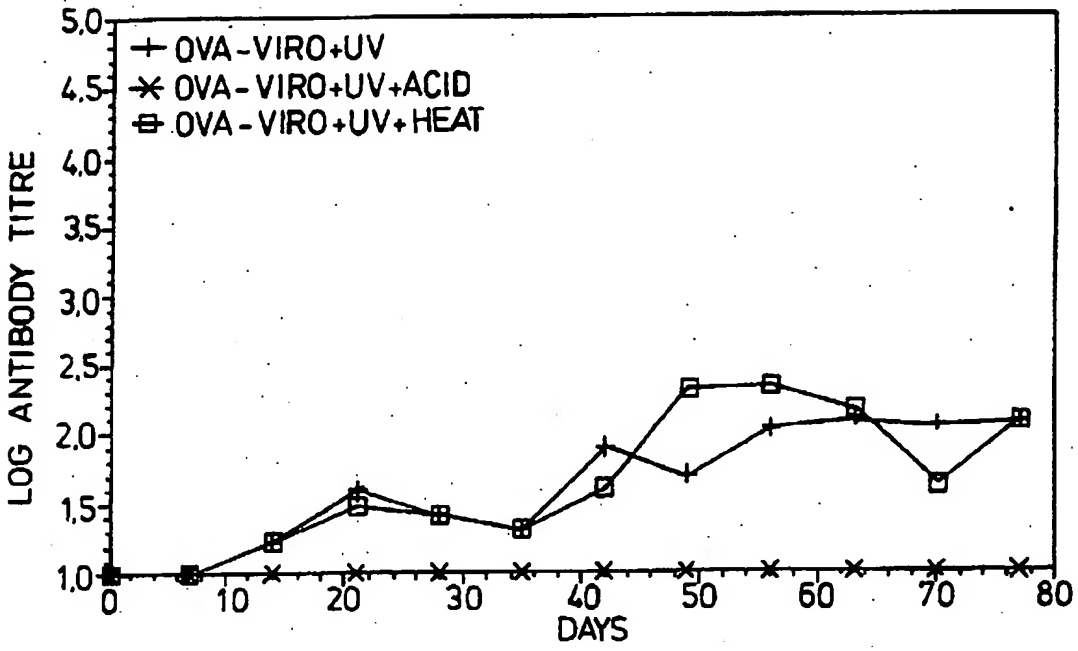
4,0
3,5
3,0
2,5
2,0
1,5
1,0
0
10
20
30
40
50
60
70
80
DAYS

Fig. 5A.


OVA-VIRO+UV
OVA-VIRO+UV+ACID
OVA-VIRO+UV+HEAT
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4.5
4.0
3.5
3.0
2.5
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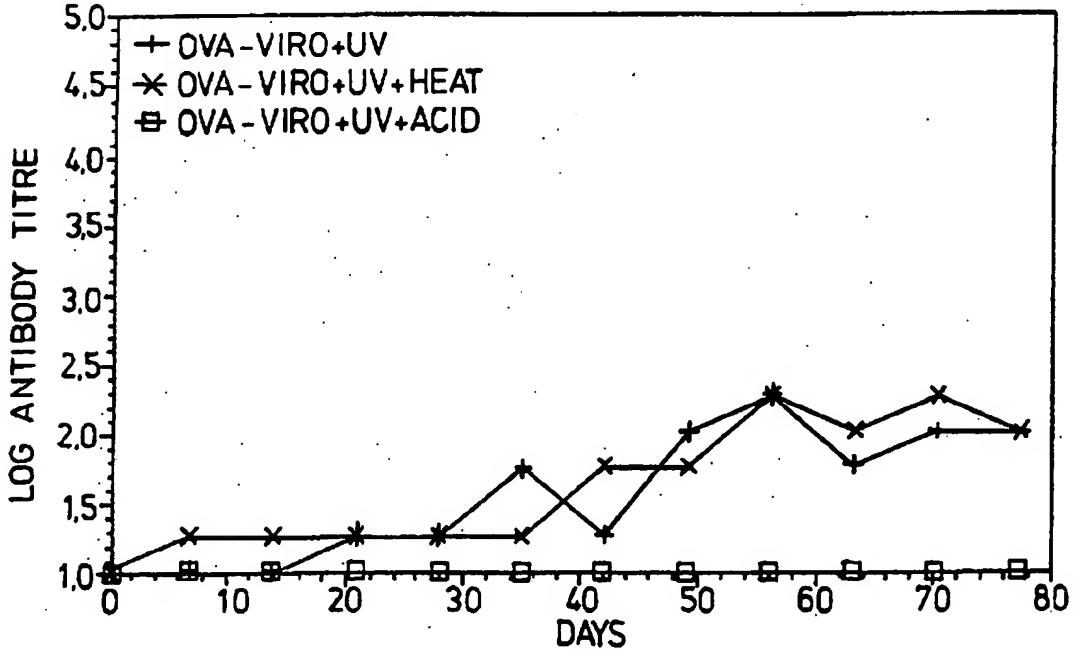
0
10
20
30
40
50
60
70
80
DAYS

Fig. 5B


OVA-VIRO+UV
OVA-VIRO+UV+HEAT
OVA-VIRO+UV+ACID
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4.0
3.5
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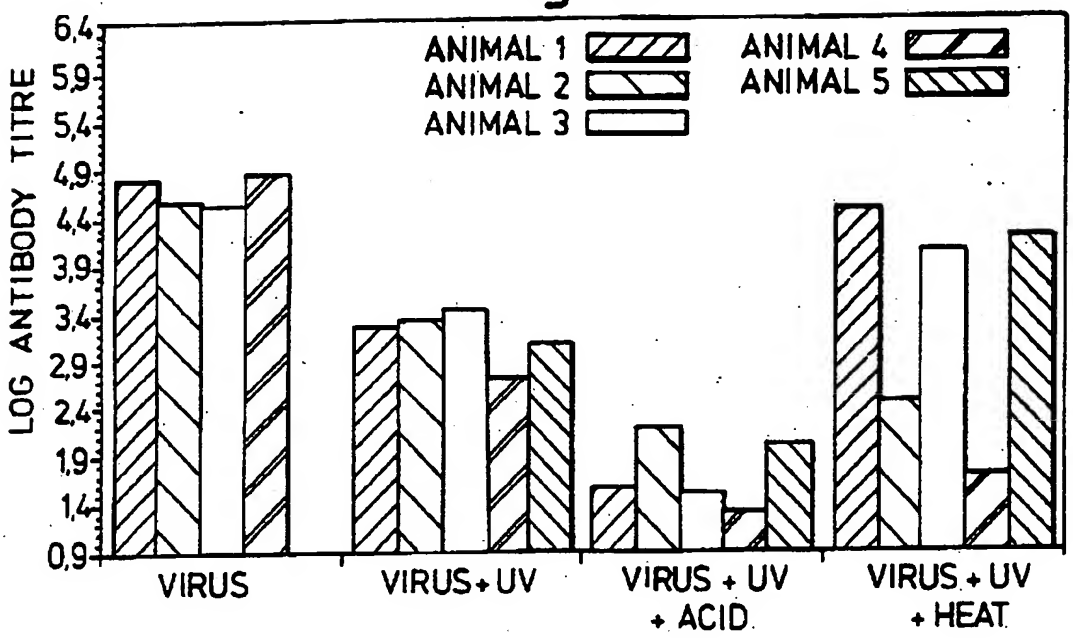
0
10
20
30
40
50
60
70
80
DAYS

Fig. 6A.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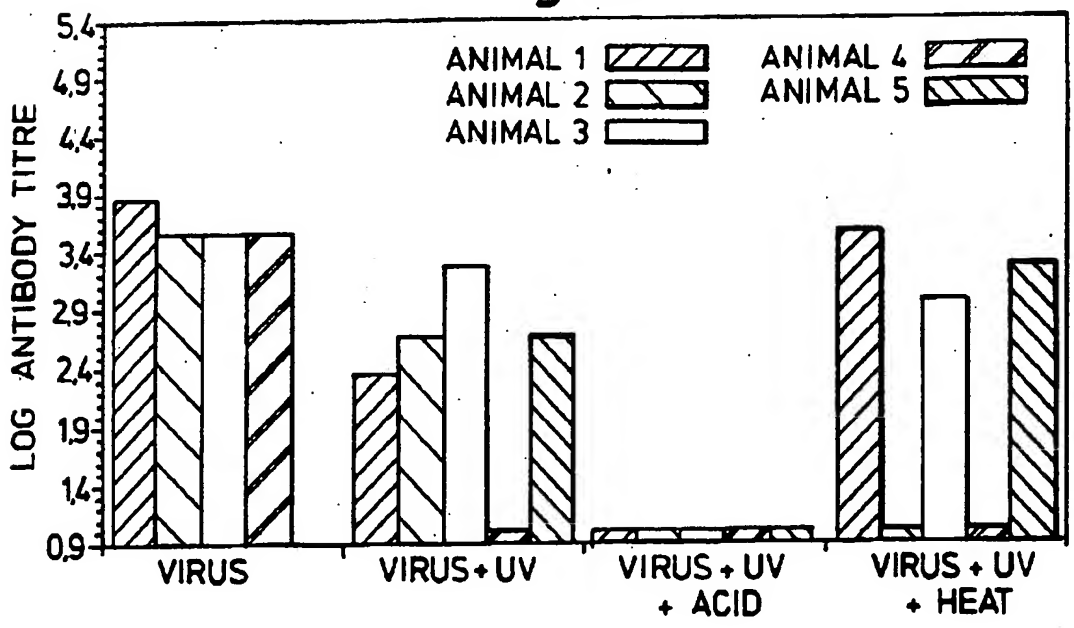
1.4
0.9
ANIMAL 1
ANIMAL 2
ANIMAL 3
ANIMAL 4
ANIMAL 5
VIRUS
VIRUS+UV
VIRUS + UV + ACID
VIRUS + UV + HEAT

Fig. 6B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ANIMAL 1
ANIMAL 2
ANIMAL 3
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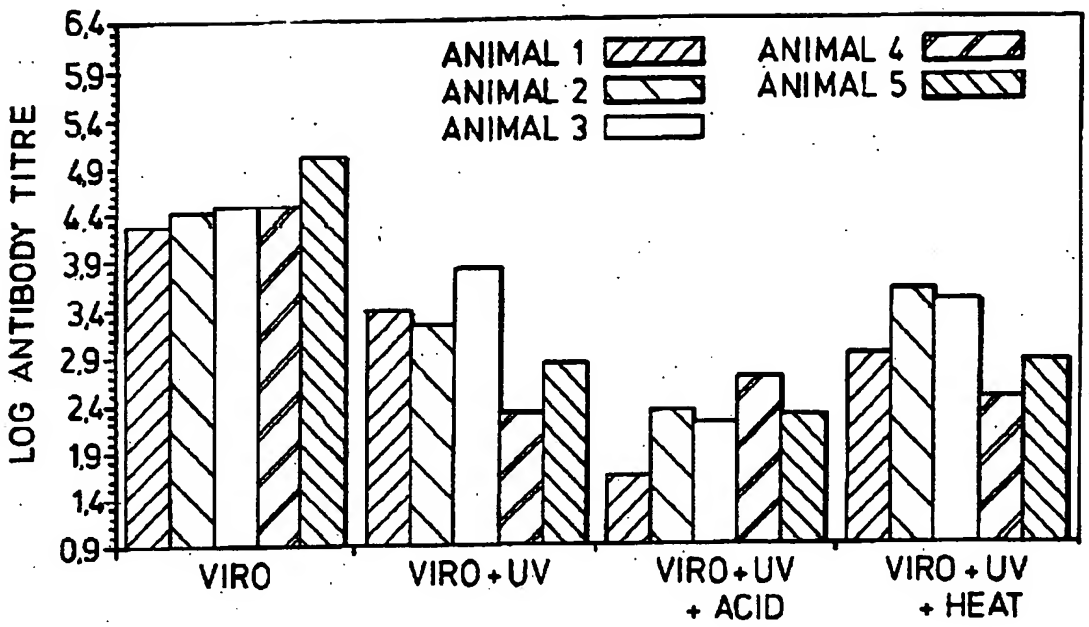
ANIMAL 5
VIRUS
VIRUS+UV
VIRUS + UV + ACID
VIRUS + UV + HEAT

Fig. 7A.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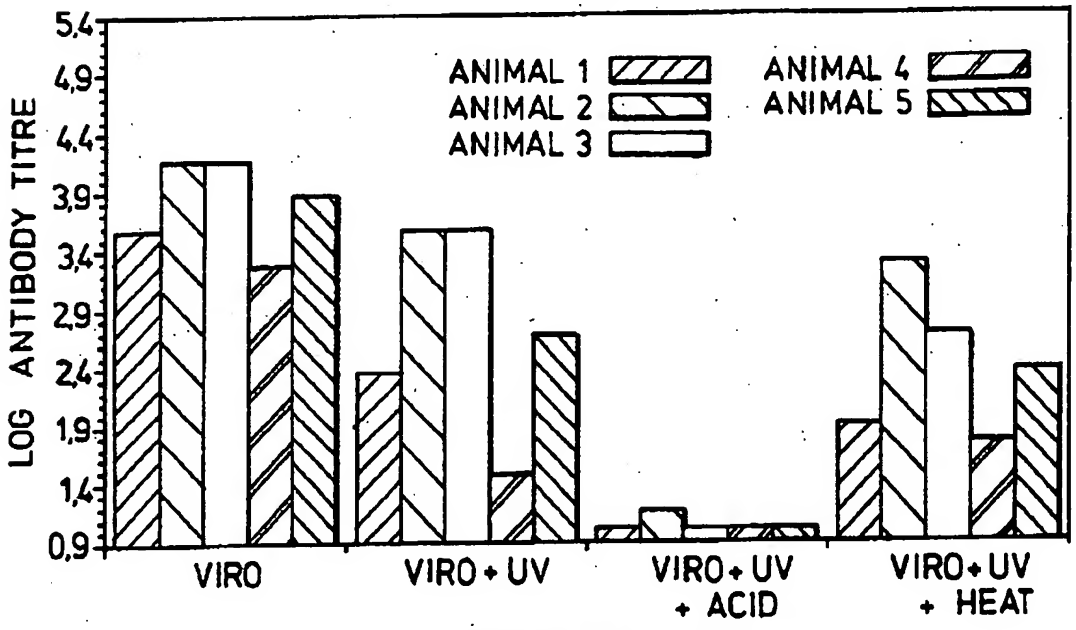
0.9
ANIMAL 1
ANIMAL 2
ANIMAL 3
ANIMAL 4
ANIMAL 5
VIRO
VIRO + UV
VIRO + UV + ACID
VIRO + UV + HEAT

Fig. 7B.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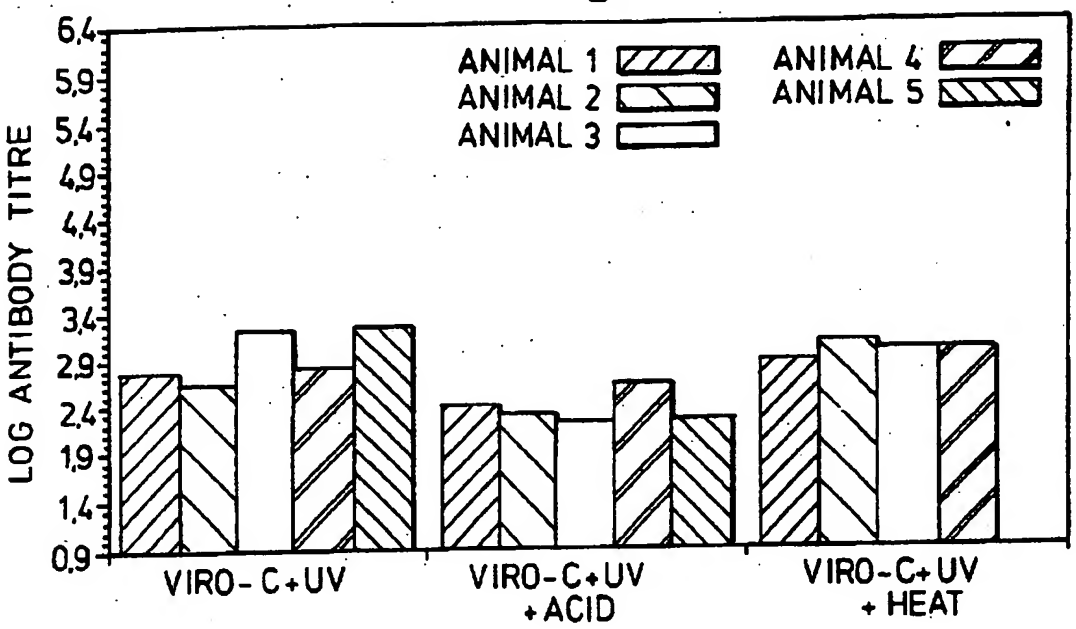
1.4
0.9
ANIMAL 1
ANIMAL 2
ANIMAL 3
ANIMAL 4
ANIMAL 5
VIRO
VIRO + UV
VIRO + UV + ACID
VIRO + UV + HEAT

Fig.8A.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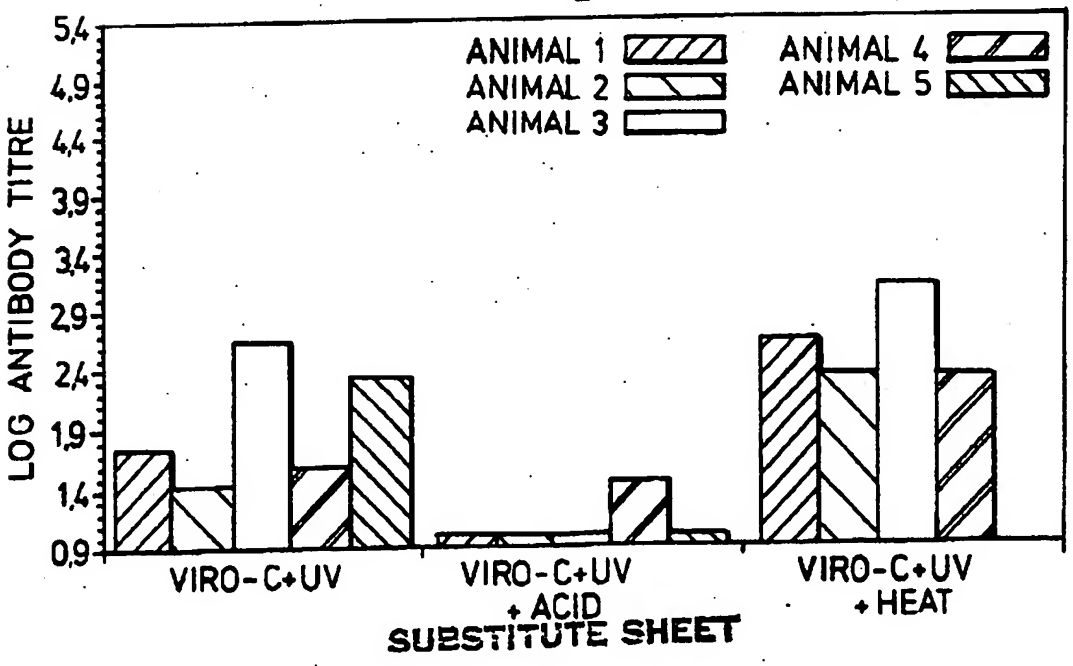
1.4
0.9
ANIMAL 1
ANIMAL 2
ANIMAL 3
ANIMAL 4
ANIMAL 5
VIRO-C+UV
VIRO-C+UV +ACID
VIRO-C+UV +HEAT

Fig.8B.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ANIMAL 2
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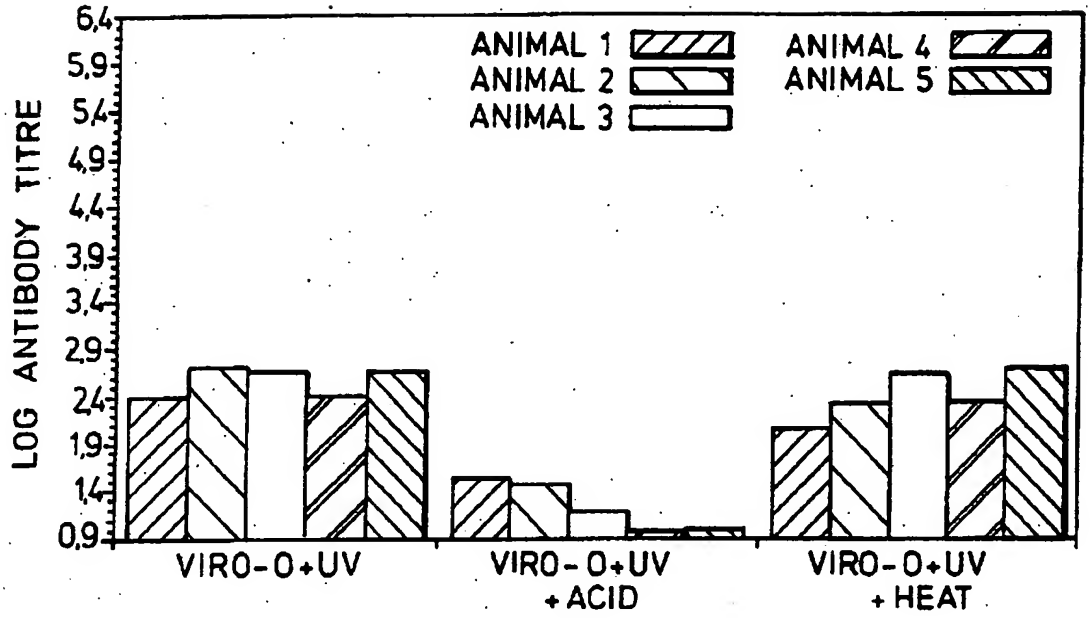
VIRO-C+UV +HEAT

Fig. 9A.


ANIMAL 1
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LOG ANTIBODY TITRE
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3,4
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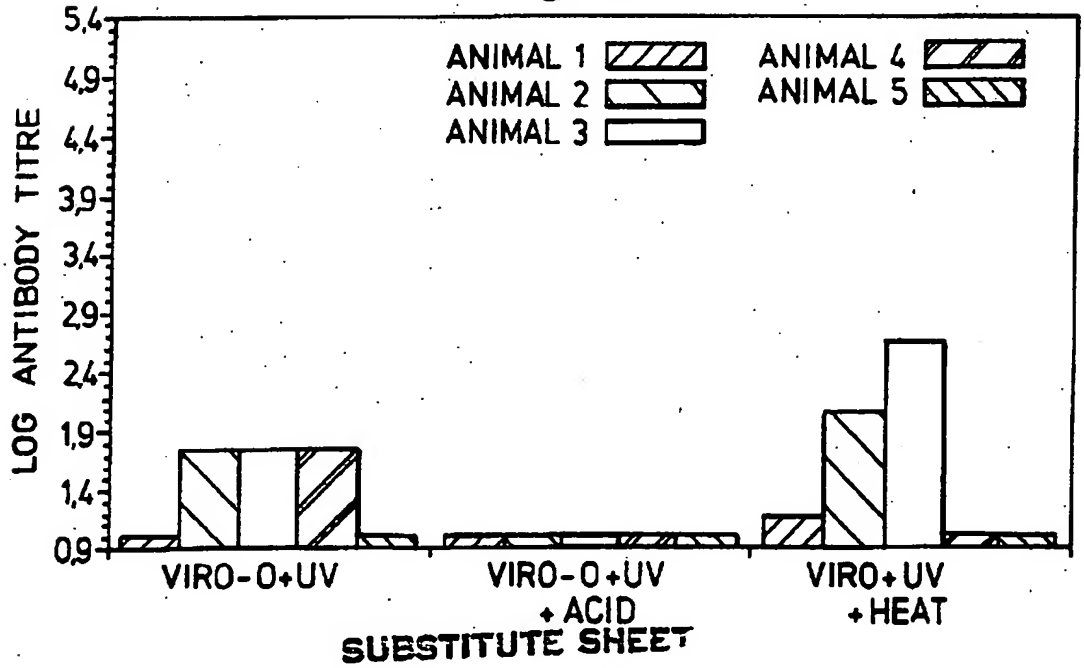
VIRO-O+UV
VIRO-O+UV + ACID
VIRO-O+UV + HEAT

Fig. 9B.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0,9
VIRO-O+UV
VIRO-O+UV + ACID
VIRO+UV + HEAT

Fig.10A

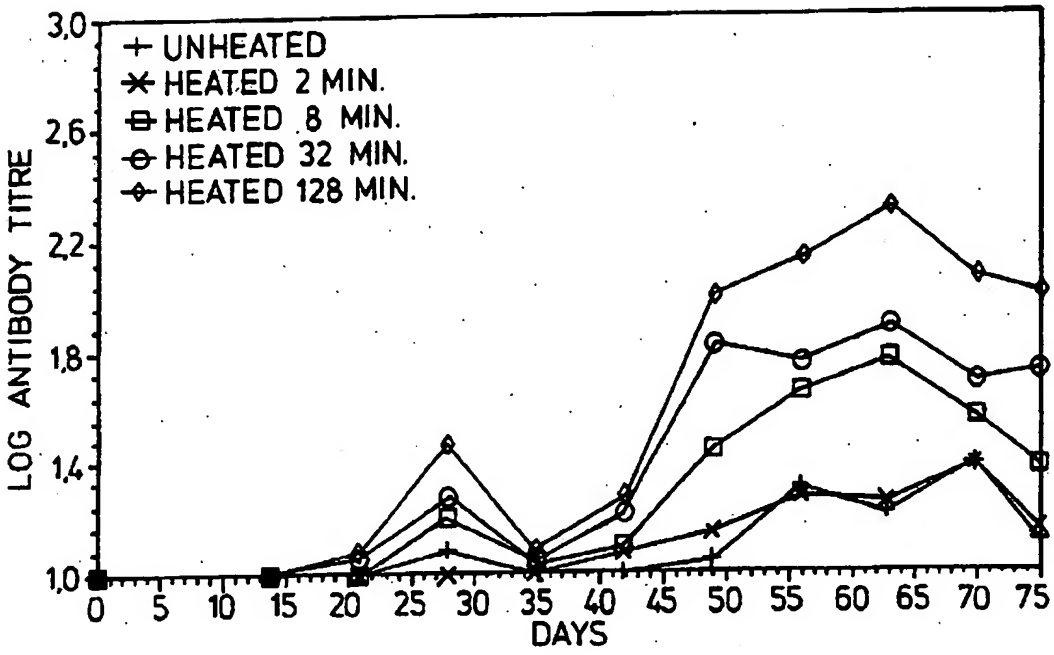


Fig.10B.

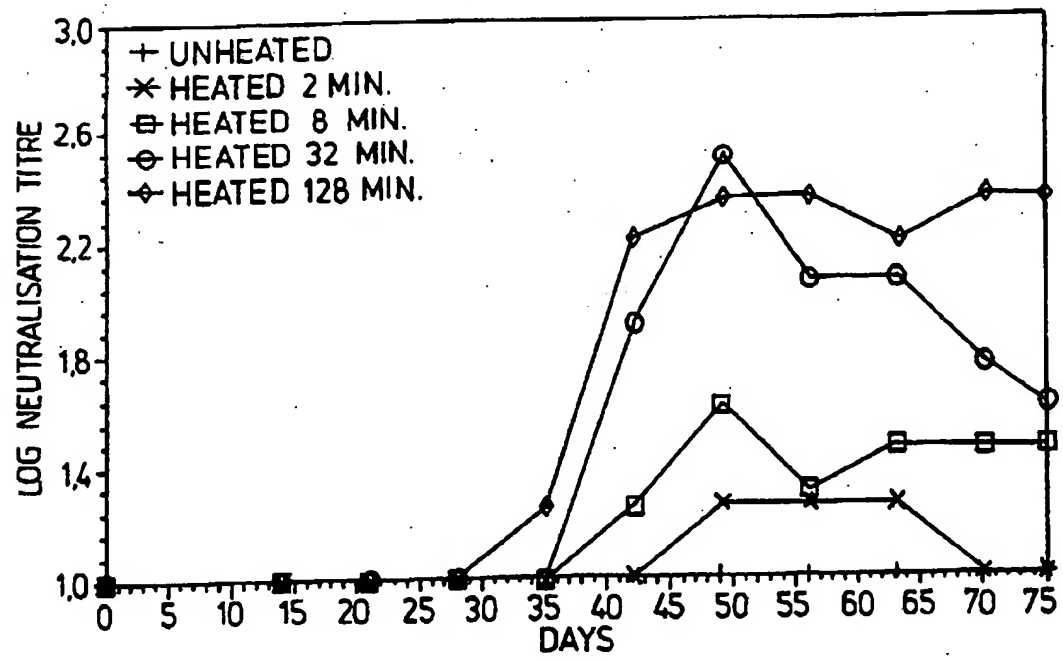


Fig.11A.

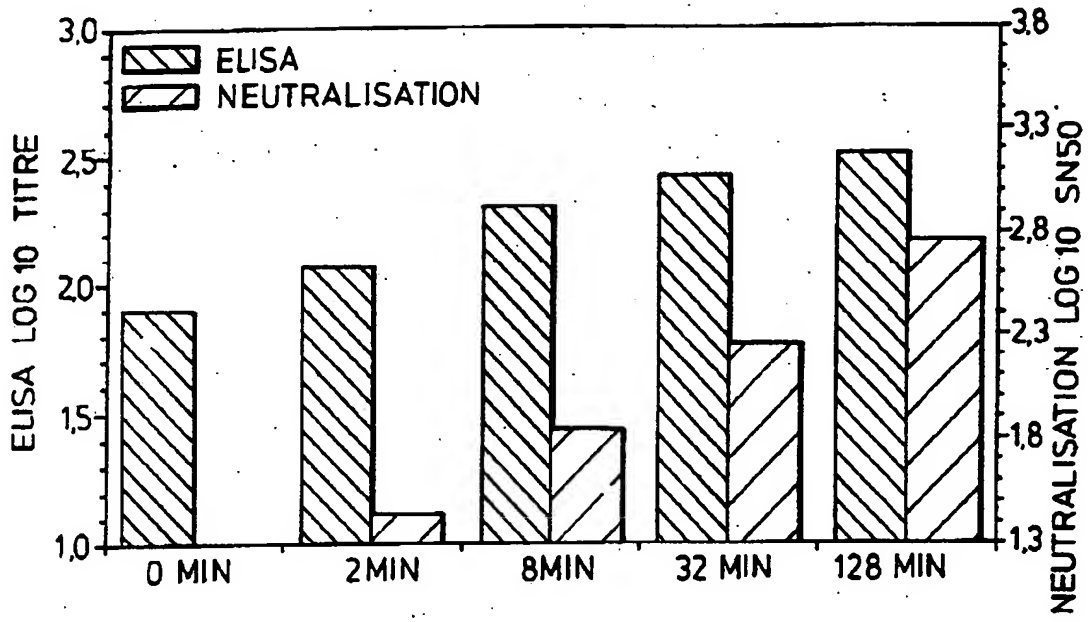


Fig.11B.

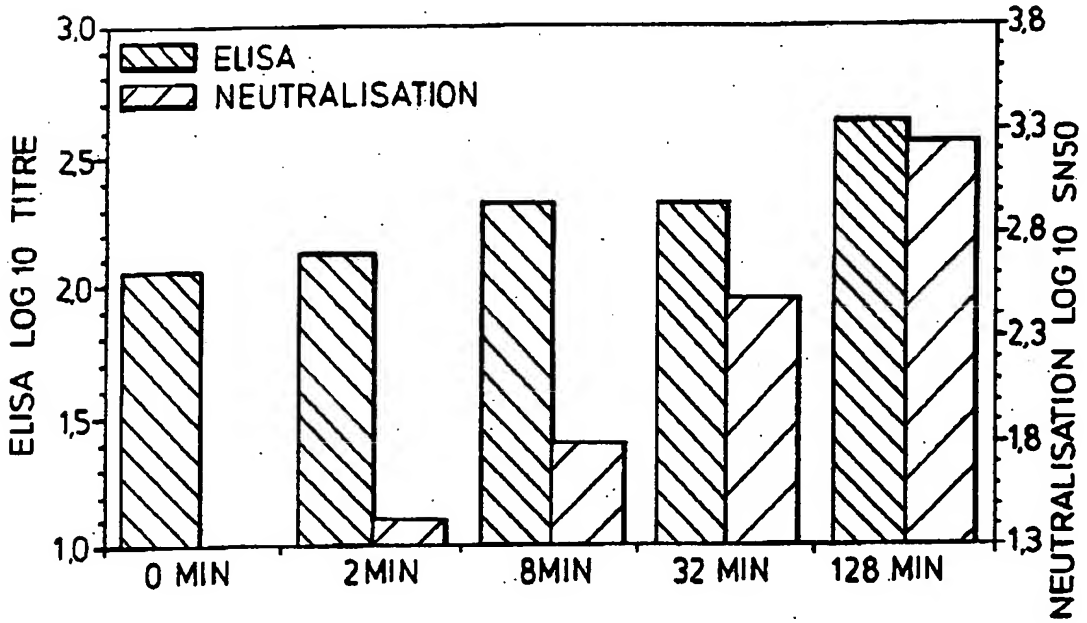


Fig.12A.

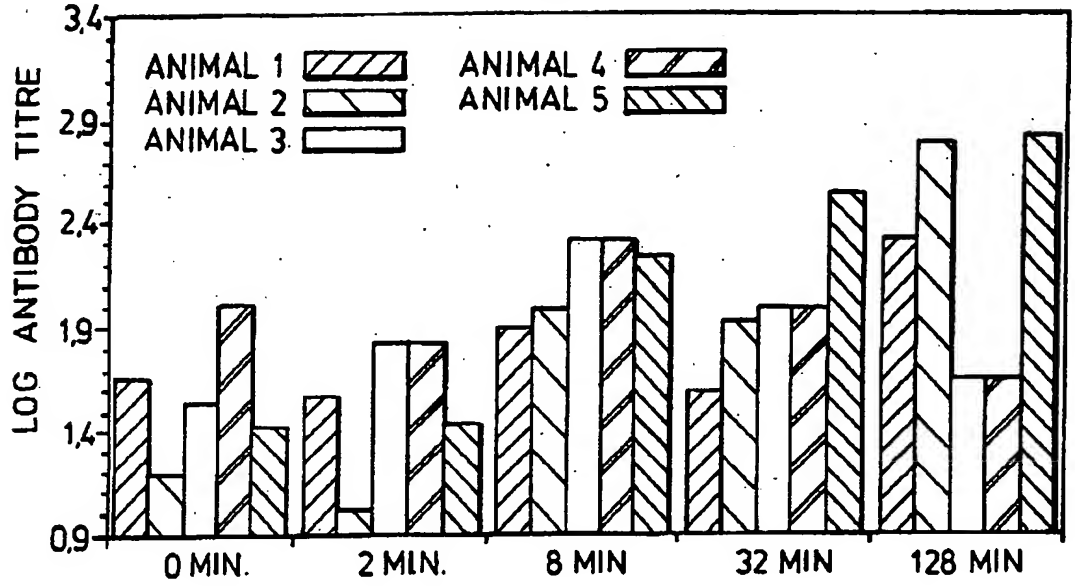


Fig.12B.

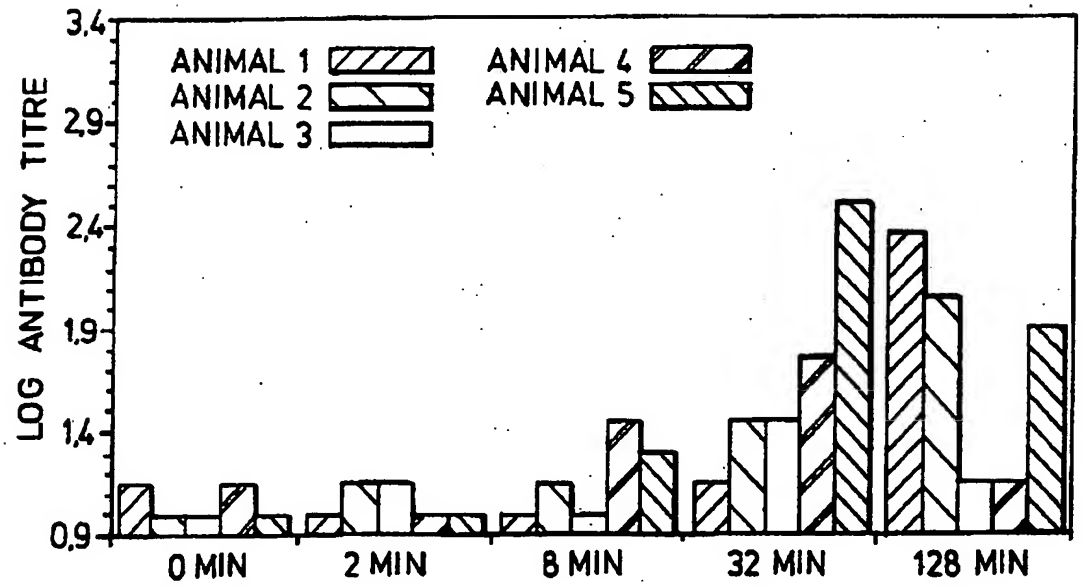


Fig.13.

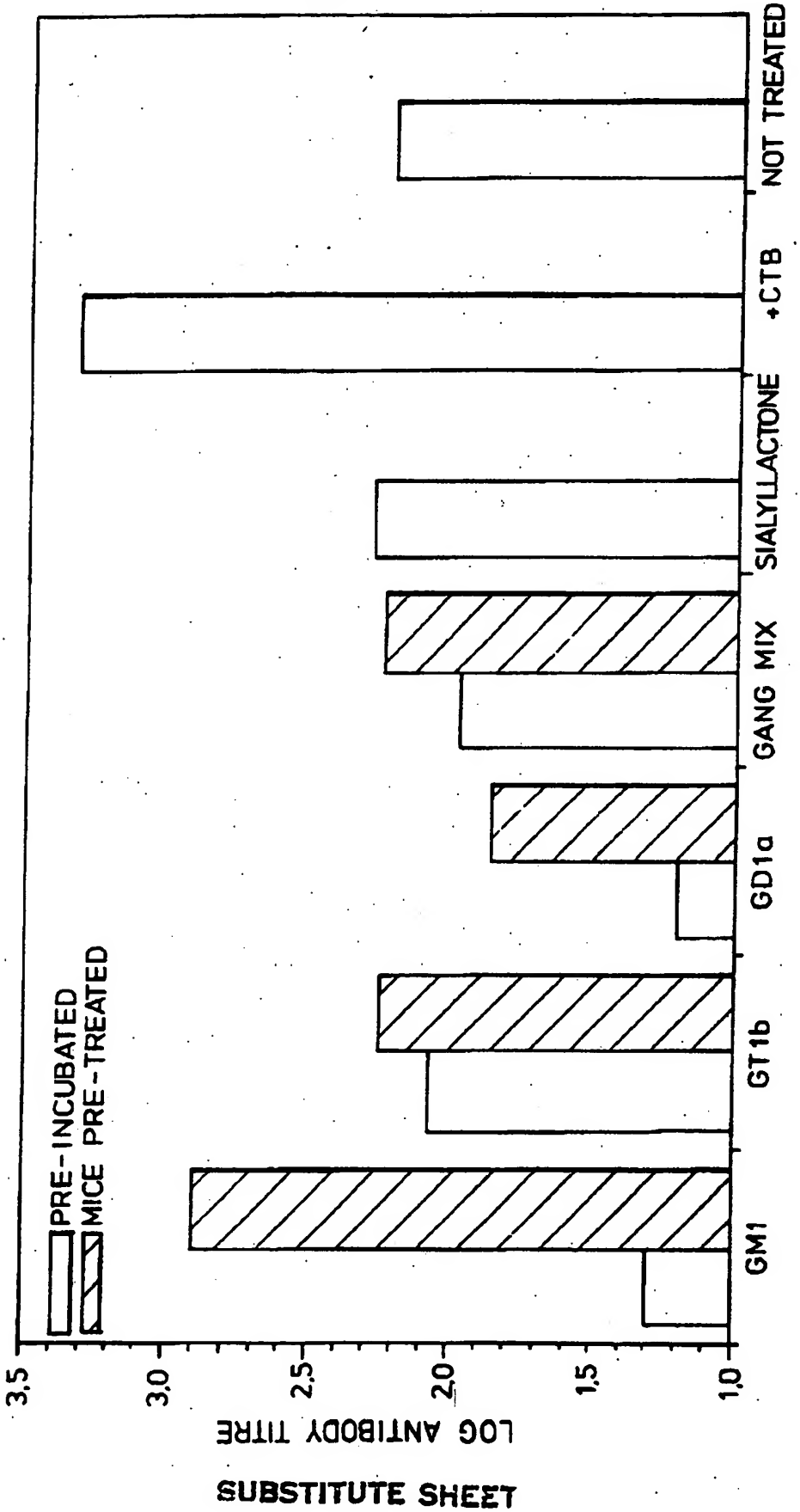

PRE-INCUBATED
MICE PRE-TREATED
NOT TREATED
+CTB
SIALYLLACTONE
GANG MIX
GD1a
GT1b
GM1
3.5
3.0
2.5
2.0
1.5
1.0
LOG ANTIBODY TITRE

Fig.14A.

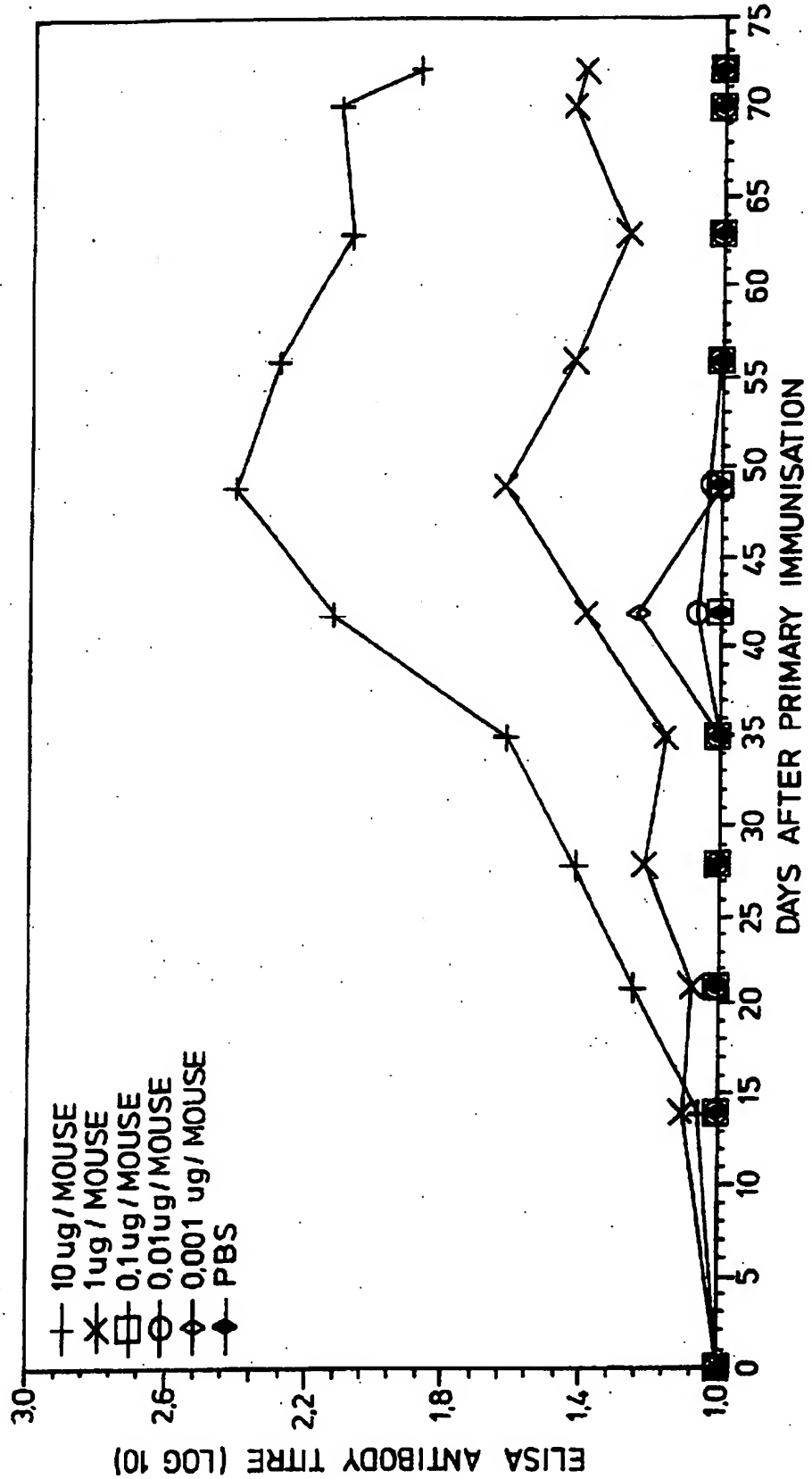
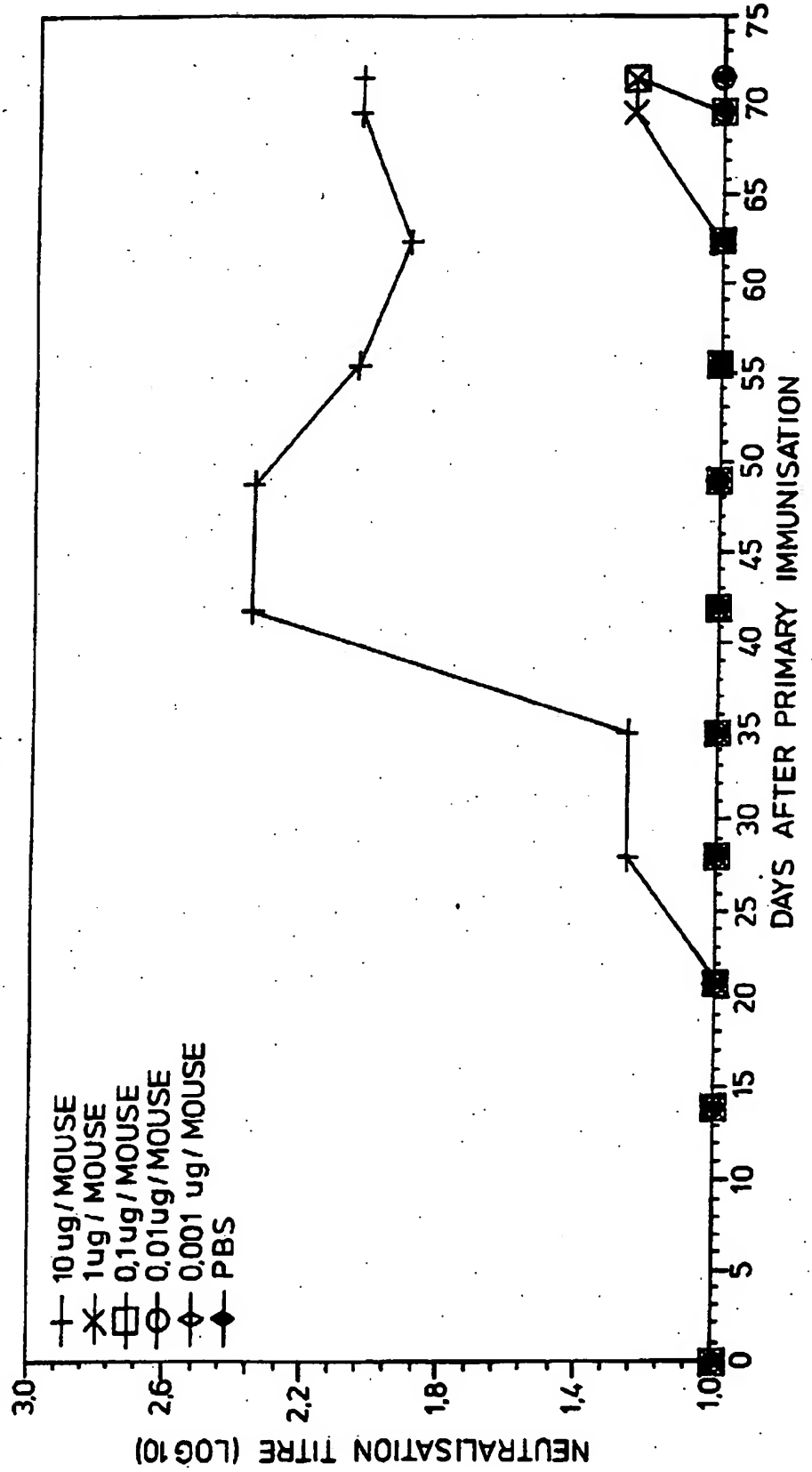

ELISA ANTIBODY TITRE (LOG 10)
3,0
2,6
2,2
1,8
1,4
1,0
DAYS AFTER PRIMARY IMMUNISATION
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
10 ug / MOUSE
1 ug / MOUSE
0,1 ug / MOUSE
0,01 ug / MOUSE
0,001 ug / MOUSE
PBS

Fig.14B.


NEUTRALISATION TITRE (LOG 10)
3,0
2,6
2,2
1,8
1,4
1,0
DAYS AFTER PRIMARY IMMUNISATION
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
10 ug / MOUSE
1 ug / MOUSE
0,1 ug / MOUSE
0,01 ug / MOUSE
0,001 ug / MOUSE
PBS

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 91/01426

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A61K39/145; A61K9/127

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category*	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X Y	CH,A,471 896 (THE WELLCOME FOUNDATION LIMITED) 13 June 1969 see column 1, line 1 - column 2, line 26	14,15,19 16-18, 20-22
Y	FR,A,2 251 334 (LENINGRADSKY POLITEKHNICHESKY INSTITUT IMENI M.I. KALININA) 13 June 1975 see page 1, line 1 - line 5 see page 5, line 36 - page 6, line 1	18,22
Y	CHEMICAL ABSTRACTS, vol. 91, no. 25, 17 December 1979, Columbus, Ohio, US; abstract no. 206999V, R.LEPRAT ET AL.: 'selective inactivation of hemagglutinin and neuraminidase on mumps virus' page 307 ;column 1 ; &arch.virol.1979,61(4),273-81 see abstract	16,17, 20,21

-/--

* Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
28 NOVEMBER 1991	16 DEC 1991
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	BENZ K.F. Benz

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	CHEMICAL ABSTRACTS, vol. 97, no. 25, 20 December 1982, Columbus, Ohio, US; abstract no. 212162G, Y.HOSAKA ET AL.: 'hemolysis and fusion by influenza viruses with heat-inactivated neuraminidase activity' page 486 ;column 2 ; &biken j. 1982,25(2),51-62 see abstract	1-13,16, 17,20,21
Y	THE EMBO JOURNAL vol. 6, no. 9, 1987; OXFORD pages 2651 - 2659; T.STEGMANN ET AL.: 'functional reconstitution of influenza virus envelopes' see the whole document document cited in the application	1-13
Y	EP,A,0 205 098 (NATIONAL INSTITUTE OF HEALTH ET AL.) 17 December 1986 see the whole document	1-13
Y	EP,A,0 356 339 (THE LIPOSOME COMPANY) 28 February 1990 see the whole document	1-13
Y	WO,A,8 808 718 (MOLECULAR ENGINEERING ASSOCIATES) 17 November 1988 see the whole document	1-13

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9101426 SA 50715

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 28/11/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

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